male germ cells of the gonad multiply and differentiate when the temperature is increased from 5 °C to 25 °C. It has been shown that cell multiplication in the albumen gland of *Helix* is linked to photoperiod [14]. It is negligible during short days (L8 : D16) but significantly higher during long days (L18 : D6).

The use of $^3$H-thymidine allowed us to show that multiplication of spermatogonia and spermatocytes is a selective response to increased temperature (25 °C) during hibernation and that the multiplication enhances with the increased length of hibernation. The weak response of SM to temperature at the start of hibernation (1 month) is without doubt one of the causes of the long delay in starting reproduction of snails at this point in their annual cycle. This state represents an adaptive mechanism which prevents onset of spermatogenesis in the event of a temporary increase in temperature during winter months. From the physiological point of view of reproduction this means that at the beginning of hibernation in snails there is a kind of refractory period comparable to that which exists in the reproductive cycle of vertebrates with photoperiod sensitivity [15]. This relative temporary insensitivity is without doubt triggered by information furnished by a decrease in the photoperiod and by unfavorable autumn environmental conditions. The factors giving rise to this decreased sensitivity of SM to temperature at the start of hibernation must come from the brain, because brain extirpation leads to a great increase in SM [16].

The purpose of this work is to show that hibernation in snails is a complex biological phenomenon during which synthesis of DNA is hardly modified by temperature in most organs (intestines, albumen gland), while in the ovotestis SM is controlled by ambient temperature which acts through the brain as an intermediary. These analyses support an increase in temperature sensitivity of SM during lengthened hibernation. This is translated by changes in the endogenous regulation of spermatogenesis during time (biological clock) in an environment where conditions are maintained constant (5 °C and permanent darkness).

The consequence of these results is important for the development of snail rearing methods because they show the need to use, as stock, animals having hibernated for 6–12 months to obtain abundant SM and rapid reproduction.

Acknowledgments. Our thanks to Prof. A. S. M. Saleuddin for help with the manuscript, to C. Colard, F. Sanchez for technical help and to B. Jolibois for typing the manuscript.

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0014-4754/90/070684-03$1.50 + 0.20/0


## Is glycogen a major energy source in avian gizzard smooth muscle contraction?

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*Received 15 September 1989; accepted 2 January 1990*

*Summary.* In comparison to other avian smooth and striated muscles, chicken gizzard contains very low levels of glycogen. This myoglobin-rich muscle seems to derive the energy for its powerful contraction from other sources.
*Key words.* Birds; gizzard; smooth muscle glycogen.

Glycogen is a readily mobilized reservoir of glucose in muscle, providing energy for contraction. The breakdown of glycogen is initiated by the enzyme phosphorylase kinase. This enzyme has been most thoroughly studied in skeletal and cardiac muscle; much less information is available about its function in smooth muscle contraction [2, 3]. Sotiroudis et al. [4, 5] have recently purified phosphorylase kinase from chicken gizzard smooth muscle; they found a very low concentration of the enzyme, which also differed from the striated muscle isoform in subunit composition and regulatory properties. In addition, the role of glycogen as an energy source for smooth

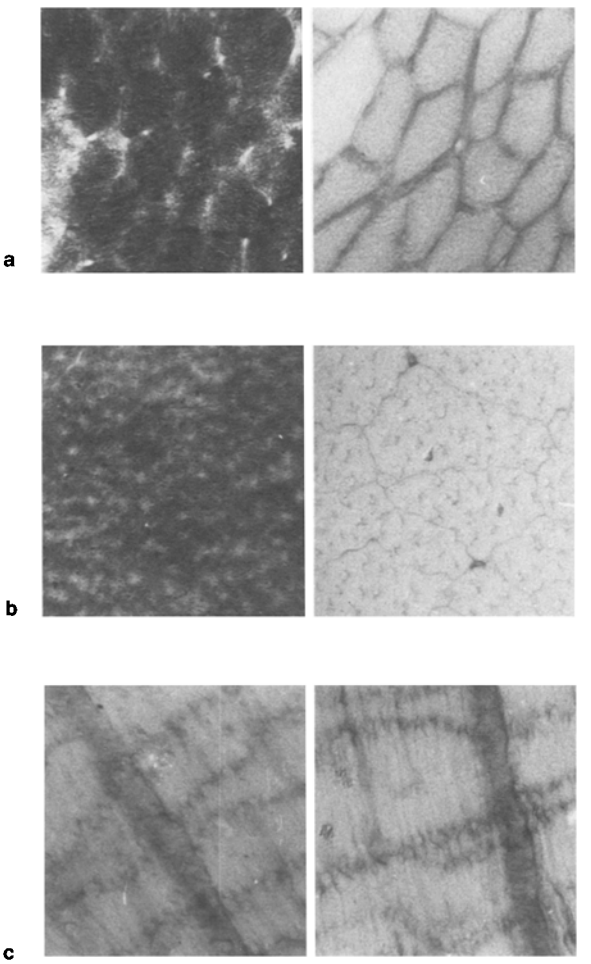
muscle contraction is still not fully understood[3]. This may in part be due to the fact that, at least in vascular smooth muscle, carbohydrate metabolism seems to be compartmentalized[6,7]. The above findings led us to measure the actual concentration of glycogen in avian gizzard muscle. Other muscle samples were also tested for comparison.

*Materials and methods*

Muscle tissue samples were removed immediately after decapitation of the birds; one sample was frozen in 2-methylbutane precooled with liquid nitrogen for histochemistry; three samples per tissue were placed in 5 vols of ice-cold 0.6% perchloric acid and homogenized. After centrifugation, the supernatants were used to assay free glucose directly, and glucose released from glycogen after incubation with amyloglucosidase (EC 3.2.1.3) for 2 h at 40 °C, pH 4.8[8]. A standard enzyme-linked spectrophotometric test (Boehringer glucose U. V., assay kit) was used. After correction for free glucose, the concentration of glycogen was calculated from the liberated glucose, using a $M_r = 162$ for anhydroglucose; the result was expressed as μmoles/g tissue (wet weight). The values represent the means of double determinations of three different samples (n = 6) ± SD. The recovery of known amounts of glycogen added to the tissue samples ranged from 97% to 98.5%. Glycogen was visualized in 10-μm fresh-frozen tissue sections attached to gelatine-coated slides by the PAS technique[9]. Controls for the specificity of the reaction were performed with sections pre-incubated with amyloglucosidase for 2 h in a humid chamber.

*Results and discussion*

The table summarizes the contents of glycogen (and free glucose) in smooth and striated muscle samples from chicken (both sedentary and free range), pigeon (flying) and duck (swimming, wings clipped). Irrespective of the bird's habitat, the gizzard samples contained very little glycogen (0.61 μmoles/g) and no free glucose. Even egg albumin, reported to have a low glycogen level[10], gave values of 1.48 μmoles/g with the method employed here.




Light micrographs of frozen sections of avian muscle stained for glycogen with the PAS reaction; before (left) and after (right) incubation with amyloglucosidase. *a* pigeon pectoral muscle; *b* chicken pectoral muscle; *c* chicken gizzard muscle.

In skeletal and cardiac muscle, originally examined for comparative purposes only, we found an interesting relationship between glycogen content and muscular activity. Thus, the pectoral and cardiac muscles of the actively flying pigeon had considerably higher glycogen values

Glycogen content of avian muscle tissue. The numbers in parentheses represent the free glucose. All values are given in μmoles/g wet weight ± SD (n = 6).

| Muscle | Bird and habitat<br>Chicken<br>(battery) | Chicken<br>(free) | Pigeon<br>(flying) | Duck<br>(swimming) |
|---|---|---|---|---|
| Gizzard | 0.61 ± 0.12<br>(0) | 0.61 ± 0.12<br>(0) | 0.61 ± 0.12<br>(0) | 0.61 ± 0.12<br>(0) |
| Uterus | 2.35 ± 0.12<br>(2.83 ± 0.22) | 2.41 ± 0.12<br>(2.94 ± 0.28) | –<br>– | –<br>– |
| Breast<br>(M.pect.major) | 5.56 ± 0.06<br>(0.33 ± 0.06) | 12.96 ± 1.23<br>(0.5 ± 0.06) | 81.48 ± 4.32<br>(3.89 ± 0.17) | 14.81 ± 0.62<br>(1.11 ± 0.11) |
| Leg<br>(M. semitend.) | 8.64 ± 0.62<br>(0.33 ± 0.06) | 11.11 ± 0.62<br>(0.67 ± 0.06) | 14.2 ± 0.06<br>(1.11 ± 0.06) | 46.9 ± 1.85<br>(3.33 ± 0.11) |
| Heart<br>(ventricle) | 1.48 ± 0.06<br>(0.11) | 7.41 ± 0.49<br>(0.06) | 24.69 ± 0.62<br>(0.56 ± 0.06) | 11.73 ± 0.5<br>(0.22) |

than were found for the muscles of the swimming duck and the free-range chicken, and especially of chickens restrained in an egg-laying battery were lowest of all. In contrast, the leg muscles of the swimming duck contained more glycogen than the leg muscles of pigeon and chicken.

Three examples of the histochemical determination of glycogen are shown in the figure. There is full agreement with the in vitro measurements. The most intensely stained was the pigeon pectoral muscle (fig., a, left), followed by the chicken pectoral muscle (fig., b, left), and in neither case did staining occur after treatment with amyloglucosidase (fig., a and b, right). There was no noticeable staining in the gizzard muscle proper; only the connective tissue septa contained PAS positive material (presumably collagen). This staining was not affected by amyloglucosidase treatment (fig., c, left and right).

The low levels of glycogen in the avian gizzard smooth muscle may not be representative of smooth muscle in general. The only other smooth muscle we examined, chicken uterus, had almost four times as much glycogen as the gizzard (2.41 μmoles/g); Lynch and Paul[6] found 2.82 ± 0.29 μmoles/g in porcine arterial muscle. It is possible that the gizzard muscle, distinguished from all other smooth muscle by its high content of myoglobin[11], derives the energy for its powerful contractions from sources other than glycogen. The differences in the isoform of phosphorylase kinase present[4,5], and the low levels of glycogen measured by us speak for this theory.

1 Support by the Deutsche Forschungsgemeinschaft (Ste 105/25-2) is gratefully acknowledged.
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0014-4754/90/070686-03$1.50 + 0.20/0


## Isolation, characterization, and determination of human liver (copper/zinc) metallothionein

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*Received 30 October 1989; accepted 13 February 1990*

*Summary.* A copper-containing protein was purified from the liver of a patient with primary biliary cirrhosis by a combination of gel filtration and anion exchange chromatography. This copper-protein had UV absorption and emission spectra, an amino acid composition, and a molecular mass which were characteristic for metallothionein (MT).

From 8 livers (3 control, 1 fetal and 4 primary biliary cirrhosis) MT was extracted with non-reducing buffer and centrifuged, and the pellets were re-extracted with a 1 % 2-mercaptoethanol-containing buffer. The non-reducing buffer extracted a predominantly copper-containing MT from the livers of patients with primary biliary cirrhosis and a predominantly zinc-containing MT from control livers and the fetal liver. Only from the fetal liver was a copper/zinc containing MT solubilized during the re-extraction with 2-mercaptoethanol-containing buffer. These results indicate that human MT is a unique metalloprotein with age and disease-dependent characteristics.
*Key words.* Metallothionein; liver; primary biliary cirrhosis; copper; zinc.

Copper is an essential trace element which is, however, potentially toxic when present in large quantities. The liver plays a key role in the homeostasis of copper and prevents accumulation of the metal by excreting the surplus into the bile. Most of the liver copper is present in the cytoplasm of the hepatocytes, and it is associated with a number of proteins. The smallest of these proteins have been identified as metallothioneins (MTs) (for review see Winge[1]). MTs have been identified as the major copper binding proteins in the livers of fetusses and newborns[2-4] and in the livers of copper-loaded rats[5] and copper-loaded pigs[6].

MTs are a group of closely related proteins characterized by an unusually high cysteine content and the absence of aromatic amino acids. The molecular mass of MTs, as calculated from the amino acid sequence, is about 6,300 Da[7], but the proteins elute at a 10 kDa position from gel filtration columns[3,8,9], and in SDS-PAGE they