intervals (Fig. 2, B3). The rapid bursting during REM episodes are illustrated by the distribution of interburst intervals, showing clusters of intervals at 200 to 400 msec (upper histogram in Fig. 2, B3). In other LC cells the relation between unit bursts and PGO monophasic waves was not maintained throughout the entire period of paradoxical sleep. The frequency of occurrence for unit bursts increased approximately by a factor of 100 from slow wave sleep to paradoxical sleep. The firing rate in the attentive wakefulness phase was even higher, ranging from 6 to 12 per second.

The entire group of 24 LC units showed comparable mean discharge rate (per second) during quiet waking (4.5 $\pm$ 1.1, S.E.) and slow wave sleep (4.2 $\pm$ 1.1) (*20*). The unit activity during paradoxical sleep was higher (10.0 $\pm$ 1.3) but similar to that in the attentive waking state (11.6 $\pm$ 1.9, $N =$ 10). During transition from slow wave sleep into paradoxical sleep, 40 percent of LC neurons slightly increased their activity, whereas 60 percent of them showed either a slight decrease or no change in firing rates. During paradoxical sleep, about 60 percent of LC units exhibited bursting behavior that was closely related to PGO spikes, or eye movements, or both. The burst discharge was more commonly seen in neurons in the medial part of the LC nucleus. During transition from paradoxical sleep to waking, 37 percent of LC neurons continued to increase their firing activity, 45 percent decreased, and 18 percent did not have apparent changes. Even those neurons whose activity decreased during this transition phase still fired faster than in quiet waking and slow wave sleep. Nevertheless, differences in neuronal discharge between various sleep periods were far less obvious from comparisons of mean discharge rate than from comparisons of interspike interval histograms.

From these experiments it appears that LC neurons become relatively inactive once the cat begins to ignore its surroundings. The inactive state of LC cells in quiet waking continues to persist through slow wave sleep until the cat goes into paradoxical sleep or else awakens and becomes attentive again. The enhanced neuronal activity in the attentive waking state may be related either to the maintenance of the state (*9*) or to behaviors that occur only during that state. Unfortunately the present recording technique has not yet allowed us to correlate unit activity with other important spontaneous behaviors such as drinking, eating, exploration, aggression, or fear.

In any event, our data indicate that NE-containing LC neurons do change their discharge patterns with sleep-waking behavior, becoming more active in attentive wakefulness and exhibiting bursting discharges in paradoxical sleep.

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19. For Prussian blue reaction and histological staining, the freeze-dried brain tissue, whose paraffin was removed, was immersed for 5 minutes in 10 percent formalin-Ringer solution which contained 3 percent potassium ferrocyanide and 3 percent potassium ferricyanide. The blue spots thus formed were counterstained for 15 to 20 minutes with 0.1 percent nuclear fast red solution or Nissl stain.
20. The statistical difference between slow wave sleep and either paradoxical sleep or attentive waking is 0.05 to 0.02 in P value (t-test), whereas the difference between quiet waking and either paradoxical sleep or attentive waking is 0.1.
21. We thank Y. Sheu for his contribution in the recording setup, B. J. Hoffer for comments on the manuscript, and S. Pekarik for secretarial assistance.

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Diazacholesterol Myotonia: Accumulation of Desmosterol and Increased Adenosine Triphosphatase Activity of Sarcolemma

Abstract. *Myotonia induced in rats by 20,25-diazacholesterol is accompanied by accumulation of desmosterol in serum, fragmented sarcoplasmic reticulum, and sarcolemma. Activities of (Na^+,K^+)- and Ca^{2+}-stimulated adenosine triphosphatases of the sarcolemma are increased, but not the Mg^{2+}-stimulated adenosine triphosphatase. The altered sterol composition of the sarcolemma may cause this type of myotonia by decreasing the chloride conductance of the membrane.*

20,25-Diazacholesterol, an inhibitor of the cholesterol synthesis at the step of the reduction of the 24-25 double bond in the side chain, induces myotonia in humans as well as in animals (*1*). The myotonia observed is similar to that of myotonia congenita and myotonic dystrophy of humans which are characterized by an impaired relaxation of skeletal muscles after contraction.

Certain features of experimental myotonia, myotonia congenita, and myotonic dystrophy suggest that a basic abnormality is located in the muscle cell membrane, which is hyperirritable and characterized by curare-resistant bursts of repetitive depolarization after mechanical or electrical stimulation [see (*2*) and (*3*)]. The accumulation of desmosterol during development of 20,25-diazacholesterol-induced myotonia in the

plasma, erythrocytes, and, to a lesser extent, in muscle of goats was described by Burns *et al.* (*1*). Our studies show that increased activities of the Ca^{2+}- and (Na^+,K^+)-stimulated adenosine triphosphatases accompany the replacement of cholesterol by desmosterol in the outer membrane (sarcolemma) of muscle from diazacholesterol-treated rats.

Female Wistar rats, weighing about 250 g, were given daily doses of 10 mg of 20,25-diazacholesterol (Searle) by esophageal canula. After 3 to 4 months of treatment, myotonia was documented by electromyography. Pairs of treated and control animals were anesthetized with ether and killed by aortal puncture.

Sarcolemma was prepared according to the method of Peter (*4*) and fragmented sarcoplasmic reticulum according to that of Worsfold and Peter (*5*). Lipids extracted from freeze-dried samples of muscle homogenates, fragmented sarcoplasmic reticulum, and sarcolemma were further washed according to Folch *et al.* (*6*). Thin-layer chromatography and densitometry were performed as previously described (*7*). Total cholesterol content was also determined chemically by the methods of Zak, with use of the correction factor for desmosterol worked out by Winer *et al.* (*1*). The esterified sterol fraction was saponified with 2*N* KOH in 95 percent ethanol at 85°C for 2 hours and the free sterols were extracted into hexane. Gas-liquid chromatographic analysis for desmosterol was performed with the use of an OV-17 column at 240°C. Values are expressed as area percentage of the total area under the peaks, assuming an equimolar response of the flame ionization detector. The peak area was determined by manual triangulation and by an automatic integrator. Adenosine triphosphatase activities of the sarcolemma were assayed as described previously (*4*).

The animals usually started to become myotonic clinically and electromyographically after about 6 weeks of feeding. Their appearance conformed to that described in the literature (*8*), but the difference in body weights never exceeded 15 percent when the animals were killed after 3 to 4 months of treatment.

In accordance with results given by Winer *et al.* (*1*) for goats, the total sterol content in the serum decreased slightly from an average of 80 ± 7 to 65 ± 8 mg/100 ml ($N = 6 \pm$ standard error) in the diazacholesterol-treated rats. The cholesterol content was greatly reduced with treatment, and desmosterol constituted 95 ± 2 percent ($N = 6$) of the total serum sterol fraction. About the same proportion of desmosterol was found in the esterified sterol fraction, ranging from 92.9 to 95.5 percent ($N = 3$). The ratio of free to esterified sterol was not significantly altered.

Table 1. Adenosine triphosphatase activities of sarcolemma isolated from normal rats and rats with diazacholesterol-induced myotonia. Values are expressed in micromoles of P_i per milligram of sarcolemmal protein per hour ± standard error of the mean. Eight normal and eight myotonic preparations were used.

(Na^+,K^+) adenosine triphosphatase	Ca^{2+} adenosine triphosphatase
Normal	
4.8 ± 1.8	24.0 ± 2.4
Myotonic	
12.0 ± 2.4	33.0 ± 2.4

Desmosterol as a percentage of total sterols averaged 83.8 percent in muscle homogenates, 81.5 percent in the fragmented sarcoplasmic reticulum, and 69.1 percent in the sarcolemma ($N = 3$; $P < .02$ for fragmented sarcoplasmic reticulum versus sarcolemma) in the treated rats. Desmosterol was barely detectable in the same fractions of the controls. The ratio of free to esterified sterol was not altered in muscle homogenates, fragmented sarcoplasmic reticulum, or sarcolemma, and the percentage of desmosterol was almost identical for free and esterified sterols.

Replacement of cholesterol by desmosterol was the only significant difference found in the lipids from myotonic rats and those from control rats. In the fragmented sarcoplasmic reticulum, as well as in the sarcolemma, the neutral lipid and the phospholipid content and their subfractions were identical and correspond to our recently published data (*7*). No significant alteration in the fatty acid pattern was obtained in the sarcolemma in contrast to the report of Seiler and Kuhn (*9*) on fragmented sarcoplasmic reticulum from animals given similar treatment. In addition, traces of esterified and dehydrogenated 20,25-diazacholesterol previously reported in the fragmented sarcoplasmic reticulum of myotonic rats (*10*) could not be detected in the sarcolemma, although as little as 1 μg of 20,25-diazacholesterol esterified with palmitate was easily detected when added to the normal muscle homogenate or the lipid extract.

Sarcolemmal membranes (Table 1) isolated from rats rendered myotonic with diazacholesterol showed significant increases in activity of (Na^+,K^+)-stimulated ($t = 2.4$; $P < .05$) and Ca^{2+}-stimulated adenosine triphosphatases ($t = 2.65$; $P < .02$) but showed no change in the Mg^{2+}-stimulated adenosine triphosphatase ($P > .3$). The yields of sarcolemmal protein from control rats were slightly but not consistently higher than those from the treated group.

Our studies show that desmosterol constitutes the bulk of free and esterified sterols in the serum and muscle of rats treated with diazacholesterol. The desmosterol content of sarcolemma isolated from these animals is only 69 percent of the total sterols compared with about 83 percent in the muscle homogenates and fragmented sarcoplasmic reticulum and 95 percent of the serum sterols. Among other possibilities, the relative retention of cholesterol by sarcolemma may reflect high affinity binding or a low turnover rate of the sarcolemmal lipid relative to the fragmented sarcoplasmic reticulum. Previous studies revealed very high concentrations of cholesterol in rat sarcolemma compared with the concentrations in fragmented sarcoplasmic reticulum or mitochondria (*7*), but the relevance of these differences to the varied functions of these membranes has not been established [see (*7*) and below].

In addition to these changes and the preservation of an otherwise normal lipid profile of the sarcolemma, our data show striking increases in the (Na^+,K^+)-stimulated and the Ca^{2+}-stimulated adenosine triphosphatases of sarcolemma in the absence of any change in its Mg^{2+}-stimulated adenosine triphosphatase. Whether these differences represent a change in kinetics of the same amount of enzyme or a net increase in enzyme units in the membranes from myotonic rats was not determined. Hence, the significance of the increased membrane adenosine triphosphatase activities and their relation to the accumulation of desmosterol in the same membranes can only be conjectured. The data clearly show, however, that the treatment with diazacholesterol not only has profound effects on the sterols of the muscle membrane but also has selective effects on its enzyme activities. The enzymic changes are not likely to be a direct consequence of diazacholesterol accumulation in view of our failure to detect the drug in muscle homogenates

or sarcolemma and because addition of diazacholesterol to isolated sarcolemma has no effect on its adenosine triphosphatase activities. Possibly the selective increases of the (Na^+,K^+)- and Ca^{2+}-stimulated adenosine triphosphatases represent a compensatory reaction to the influx of Na^+ and efflux of K^+ associated with the repetitive action potentials that characterize myotonic muscle.

The specific membrane resistance is more than doubled in diazacholesterol-treated rats (*3*). This increase was expected to reflect a decrease in chloride conductance because chloride conductance is two- to threefold that of potassium in normal mammalian muscle (*11*). Indeed a recent study (*12*) showed that chloride conductance is only one-third of normal in diaphragm fibers of diazacholesterol-treated rats. We suggest that in diazacholesterol-treated rats the abnormal sterol composition of the sarcolemma results in decreased chloride conductance and thus accounts for the repetitive action potentials and the resultant delay in relaxation of myotonic muscle. This hypothesis is consistent with the observation that chloride conductance induced in thin lipid films by certain polyene antibiotics is dependent on the sterol composition of the film (*13*). Similar mechanisms may apply to certain forms of human myotonia, including myotonia congenita in which increased membrane resistance and decreased chloride conductance are recognized (*14*).

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Fate of the Nucleus of the Marrow Erythroblast

Abstract. *Nucleated red cells lose their nuclei during passage through the endothelium of marrow sinuses. The passage occurs through cytoplasmic pores which are not gaps at the junction of two endothelial cells but perforations within the endothelium. Enucleation occurs because the pores are of relatively fixed size. Whereas the cytoplasm is flexible and squeezes through the pore, the nucleus is rigid and cannot conform to the pore size. It is, thus, caught, and the red cell becomes enucleated.*

The function of the endothelium of splenic sinuses in controlling transmural cellular passage is well known: rigid intracellular materials such as Heinz bodies are removed, whereas the rest of the red cell is allowed to pass by. Such a mechanism is commonly known as "pitting" (*1*). We now provide evidence that a similar mechanism is operative in cellular passage in the wall of bone marrow sinuses.

In bone marrow, hematopoiesis occurs outside the sinuses. At the stage of orthochromic normoblast polarization of the nucleus occurs so that the bulk of the cytoplasm lies closer than the

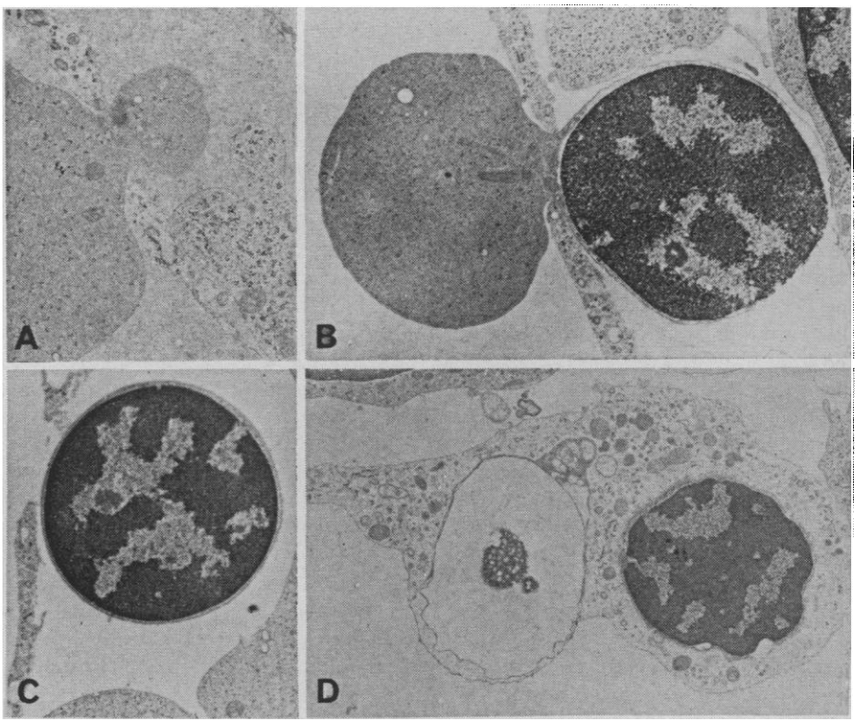

Fig. 1. Various stages of transmural passage and enucleation of red cells and mature normoblasts. (A) The cytoplasm in passage from the cord into the sinus. The size of the aperture appears to exert a control on the passing red cell, and the cell must conform to this size (× 8500). (B) Nucleated red cell in passage through the wall of a marrow sinus. The cytoplasm is completely within the lumen. The nucleus, however, cannot conform to the size of aperture and the nucleus remains behind (× 8500). (C) This nucleus, located in an area adjacent to a pore, may have been recently lost from a passing normoblast (× 8500). (D) The nucleus of a normoblast is still recognizable in the cytoplasm of this cordal macrophage. The remnant of another nucleus is seen within a phagosome (× 6800). In A, B, and C the cord is on the right and the lumen on the left.