Digestive enzymes in the alimentary canal of *B. geminata*

Enzymes	Foregut (crop) Tissue	Foregut (crop) Contents	Midgut Tissue	Midgut Contents	Hindgut Tissue	Hindgut Contents
Pepsin-like protease	–	–	–	–	–	–
Trypsin-like protease	+	+ + +	+ +	+ + +	+	+ + +
Chymotrypsin	–	+ + +	+ +	+ + +	–	+ + +
Aminopeptidase	–	+ +	+ +	+ + +	–	+ +
α-Amylase	+	+ + +	+ +	+ + +	–	+ +
Cellulase	–	–	–	–	–	–
α-Glucosidase						
a) Maltase	–	+ + +	+ +	+ + +	–	+ +
b) Sucrase	+	+ + +	+ +	+ + +	+	+ + +
β-Galactosidase						
Lactase	–	–	+	+ +	–	+
Lipase	–	+ + +	+ +	+ + +	–	+ +

ileum and rectum, both 6.7–6.8. These values differ from those reported by Mehrotra and Sen[4] for 3 species of dragon-flies in which the pH of foregut contents ranged pH 8.4–8.9 and of midgut pH 5.4–5.9.

In *B. geminata*, digestive enzymes have been found to be present in both the tissues and contents of each region of the alimentary canal (table). It will be seen that since the midgut tissue secretes 8 enzymes, this region, is the main site of enzyme secretion. Enzymes detected in tissues of the foregut and hindgut are probably endoenzymes[12]. Whereas those present in the foregut contents presumably reach there by regurgitation, a well-known phenomenon[13], and those in hindgut arrive along with the digested food. An additional enzymic source can be the alimentary canal of the preyed insect. According to the array of enzymes, *B. geminata* is capable of metabolising carbohydrates, proteins and fats, unlike *Libellula luctuosa* in which the enzyme capable of digesting starch (amylase) is absent[2].

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Use of ^{31}P NMR to measure pH of membrane-enclosed solutions

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Summary. Phosphorus nuclear magnetic resonance was employed to measure the internal pH of unsonicated multilamellar liposomes. A steady transmembrane pH gradient could be obtained in the presence of sulfate and citrate anions. The effects of some uncouplers and ionophores on this controlled pH gradient were studied.

Available methods for estimating pH of membrane-enclosed solutions include measurements of the absorbance of indicator dyes[3], the equilibrium distribution of weak acids[4] or amines[5,6] in response to pH gradients, the fluorescence quenching of freely diffusible fluorescent amines[7]. The main limitation of these techniques, when applied to living organelles, lies in their great potential for interfering with biological processes, e.g. indicator dyes are likely to participate in oxidation-reduction reactions. Such methods appear rather laborious and can only provide indirect information about internal pH.

^{31}P and proton NMR techniques offer a very simple, direct method for measuring pH gradients across biomembranes[6,8,9]. ^{31}P NMR seems in this respect more promising than proton NMR, considering the much easier interpretation of ^{31}P NMR-spectra, the number of phosphorylated metabolites that can be monitored in living cells and the high resonance resolution of this method due to the large chemical shift range.

In the present study, the potentiality of this approach was tested in a series of experiments on the proton permeability of phospholipid liposomes which were loaded with inorganic phosphate to detect the internal pH.

Multilayer liposomes were chosen for such an approach as they have a large internal volume and are readily washed by centrifugation. Multilamellar liposomes were prepared by evaporating chloroform solutions containing 75 μmoles of egg phosphatidylcholine plus 5% egg phosphatidic acid, and shaking the lipids in 3 ml of a buffer containing either 0.3 M Na_2HPO_4 or 0.3 M K_2HPO_4 at pH 7. The milky

Table 1. Proton permeability of liposomes. The liposomes loaded with 0.3 M Na_2HPO_4 at pH 7 were washed twice and suspended in 1 ml of the indicated medium buffered with 30 mM trisodium citrate as described in the methods. ^{31}P NMR-spectra were recorded at various times after the pH gradient was established. The times are the midpoints of the 500 scans spectral accumulations

External medium	pH of enclosed phosphate solution at various times				
	30 min	60 min	120 min	180 min	240 min
0.3 M Na_2SO_4 pH 5	7	7	7	7	7
0.45 M NaCl pH 5	6.7	6.1	5.6	5	5
0.3 M Na_2SO_4 + 0.1 M sodium acetate pH 5	5.1	5	5	5	5

suspension was centrifuged at 3500 rpm for 30 min and the liposomes, collected as a floating layer, were washed twice with equiosmolar Na_2SO_4 or K_2SO_4 solutions at the same pH. The lipids were finally suspended in 1 ml of the medium indicated in the legends to tables and figures containing 10% D_2O and ^{31}P NMR-spectra were recorded. All solutions were buffered with 30 mM trisodium citrate. Buffer pH was adjusted by addition of H_2SO_4.

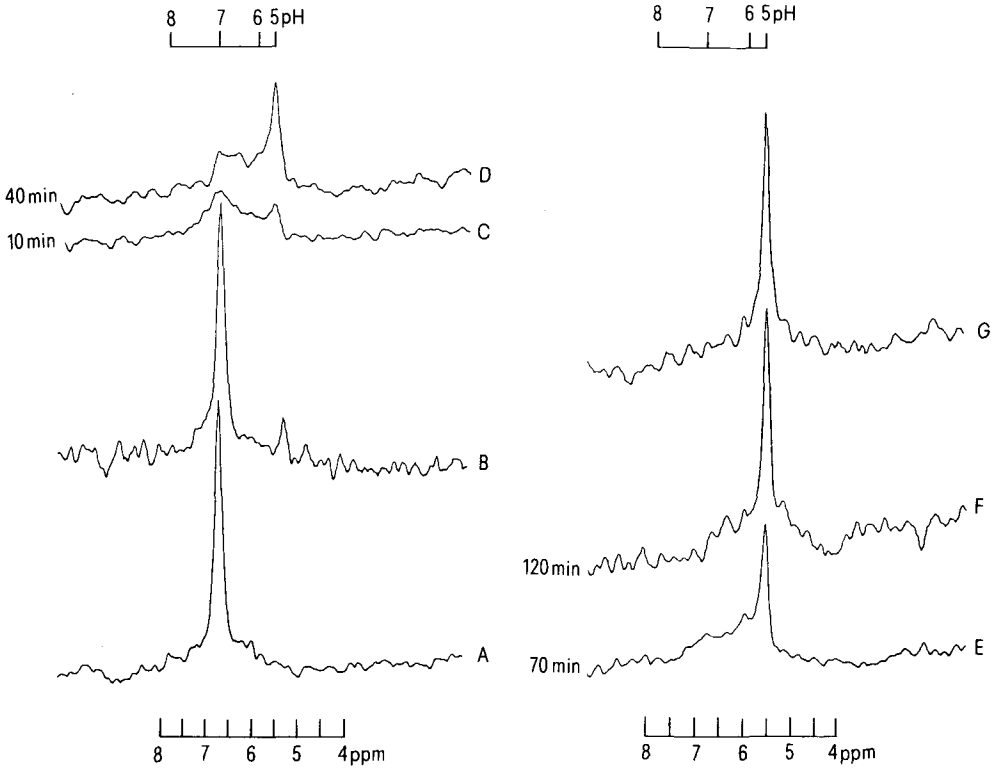
^{31}P NMR-spectra were recorded at 36.4 MHz at room temperature (20 °C) on a Bruker WH-90 Fourier transform-spectrometer. Accumulated free induction decays were obtained from 500 transient with an interpulse time of 1.7 sec, using 90° pulses. Chemical shifts were measured with respect to external triphenylphosphine in chloroform.

Under the experimental conditions used in this study (low phospholipid concentration, short accumulation time, small spectral width) the very broad signal of the phospholipid phosphate groups is not observable[10].

Results and discussion. In agreement with previous observations[11], the ^{31}P resonance of inorganic phosphate proved quite sensitive to changes in pH between 6 and 8. To rule out the possibility that a contribution to the ^{31}P resonance observed might be due to phosphate leaked out of the liposomes, 1 mM $CoCl_2$ was routinely added to the liposome suspension at the end of each experiment. No change in intensity occurred (compare, for instance, spectra F and G of the figure), whereas the resonance was broadened beyond detection when Co^{2+} was added to a similar amount of phosphate free in solution.

It is apparent from table 1 that the pH gradient was not dissipated as long as 4 h when the external electrolyte was sodium sulphate. In such conditions, no proton leakage was detectable even when the pH of the external medium was as low as 3.5 (unpublished observations). On the contrary, in the presence of sodium chloride the 2 pH unit gradient fully collapsed within 3 h. This result is in agreement with previous findings and may conceivably be due to the covalent association of protons with chloride anions at the lipid-water interface[12,13]. When sodium acetate was added to the sulphate solution, proton equilibration across the bilayers was virtually complete after 30 min. The same results were obtained when K_2HPO_4 and K_2SO_4 were substituted for the sodium salts.

The figure presents typical ^{31}P NMR-spectra of multilamellar liposomes loaded with phosphate. Lowering the pH of the external medium to 5 did not bring about any change in the chemical shift of ^{31}P resonance of internal phosphate, as the liposomes proved completely impermeable to protons for several h in such conditions (see table 1). The addition of 200 µg of gramicidin resulted in a rapid broadening of the phosphate peak over a 2-pH unit interval, followed by the appearance of an increasing peak at pH 5 and the gradual and finally complete disappearance of the phosphate signal at pH 7. This indicated that protons were



36.4 MHz ^{31}P NMR-spectra of unsonicated liposomes loaded with phosphate. The liposomes prepared in 0.3 M K_2HPO_4 at pH 7 were washed twice and resuspended in 1 ml of 0.3 M K_2SO_4 at the same pH (spectrum A). The lipids were subsequently centrifuged and resuspended in 0.3 M K_2SO_4 at pH 5 (spectrum B). Spectra C to F were recorded at various times after addition of 200 µg of gramicidin dissolved in 5 µl of ethanol. Spectrum G was recorded after addition of 1 mM $CoCl_2$. The times are the midpoints of the 500 scans spectral accumulations. Chemical shifts are given in ppm downfield external triphenylphosphine.

being transported into all the successive compartments of the multilayer structures in exchange for sodium ions. The persistence of the higher field resonance after addition of cobalt ruled out the possibility that the appearance of this peak might be due to a leakage of phosphate.

Table 2 lists the effects of various ionophores and FCCP on the proton permeability of liposomes. No appreciable change in the internal pH was detectable after addition of FCCP as the electrogenic proton translocation brought about by this uncoupler soon generates a diffusion potential which prevents, in the absence of an exchangeable cation, any further proton influx. When valinomycin, which itself had no effect, was added together with FCCP, the pH gradient was completely destroyed within 1 h. A similar but faster effect was detected in the presence of nigericin, which catalyzes an electroneutral $K^+:H^+$ exchange. The addition of gramicidin allowed a slower but likewise complete proton equilibration across all bilayers, as shown in detail in the figure. A similar effect by gramicidin could be detected when sodium salts were used.

This result is relevant to the cellular action of gramicidin because it indicates direct action of this antibiotic on the membranes of subcellular structures.

Table 2. Effect of FCCP and various ionophores on proton permeability of liposomes. The liposomes loaded with 0.3 M K_2HPO_4 at pH 7 were washed twice and suspended in 1 ml of 0.3 M K_2SO_4 at pH 5. ^{31}P NMR-spectra were recorded at various times after the addition of the indicated drug. In parentheses are indicated the times at which proton equilibration was complete

Addition	pH of enclosed phosphate solution
1 µg FCCP	7*
2 µg valinomycin	7*
2 µg valinomycin + 1 µg FCCP	5 (60 min)
2 µg nigericin	5 (20 min)
200 µg gramicidin	5 (120 min)

*No change in the pH value could be detected for several h after the addition of either FCCP or valinomycin.

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Effects of disuse and nerve stump length on the development of fibrillation in denervated soleus muscle

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Summary. Both in normal (control) and in cordotomized (disused) rats, the soleus muscle was denervated either by cutting the sciatic nerve near the trochanter (proximal denervation) or by cutting the soleus nerve near the insertion into the muscle (distal denervation). In the control muscles, the development of fibrillation was not dependent on the level of nerve section. In disused muscles, the development of fibrillation was greater following distal denervation than following the proximal one.

Spinal cord section, performed a week before denervation, accelerates the onset of fibrillation in the soleus (slow) muscle of the rat, but reduces its development, which becomes similar to that observed in the anterior tibialis (fast) muscle[1-2].

The hypothesis was made[2] that the 'fast' pattern of fibrillation development in disused and denervated slow muscle represented indirect evidence of the muscular speeding following disuse. In the same paper it was also proposed, although on a purely theoretical basis, that the earlier onset of fibrillation would slow down the subsequent development because of the persisting release of a neurotrophic factor by the peripheral nerve stump. Indeed, by several authors it has been reported that the denervation changes are affected by the length of degenerating nerve fibres[3-6].

In the present work, the possible influence of the nerve stump on fibrillation development has been investigated in the disused-denervated preparation, by cutting, in cordotomized rats, the soleus nerve at 2 different levels, and then by comparing the spontaneous activity under the 2 experimental conditions.

Methods. All surgical procedures were performed in adult albino rats, 200-250 g in weight, under ether anaesthesia. The spinal cord was sectioned at the mid-thoracic level and 7 days later the sciatic nerve was cut bilaterally near the trochanter (proximal denervation), and unilaterally near the nerve insertion into the soleus muscle (distal denervation). The difference in nerve stump length achieved by cutting at the 2 levels was about 4 cm. Distal denervation was always associated with the proximal one, with the purpose of preventing the motor activity of the other muscles of the lower leg: this activity, in fact, would have introduced differences in the mechanical conditions of the distally denervated soleus muscle, in comparison with the contralateral muscle. In another group of animals, after spinal cord section and bilateral proximal denervation, a sham distal operation was performed in order to produce approximately the same amount of trauma caused by the