Dextrose (*14*) was added to a week's supply. The females were kept on these diets for 1 month before mating and throughout gestation. The restriction was such as to still permit full-term gestation (*16*) and normal number in litter.

The newborns were weighed, and then killed by decapitation, within 6 hours of delivery. The brains (cerebral hemispheres) were immediately removed without cerebellum and olfactory lobes (*13*) and weighed; they were then frozen and subsequently used for the analysis. DNA was determined by a modification of diphenylamine colorimetric method (*12*, *17*), and protein was determined by a modification of Folin colorimetric method (*18*).

The results (Table 1) show first that the rats on two different full diets exhibited differences in body and brain weights, but the total amount of DNA [and therefore total brain cell number (*19*)] was the same. Thus, cell number is a more constant indicator; the brain weight cannot be used as a measure of brain cell number.

As expected (*2*, *6*), dietary protein restriction of the mother resulted in considerably (30 percent) lower body weights of the newborn offspring; however, in contrast to previous experiments (*2*), in which the dietary restriction was during gestation only, in our experiments in which the restriction was also imposed 1 month before mating, the brain weights were also considerably (23 percent) lower. This decrease is reflected in comparable percentage decrease in total protein content. All these changes are statistically significant.

The restriction also resulted in a significantly lower (10 percent) DNA content, that is, significantly lower total brain cell number. However, this difference is less pronounced than the difference in brain weight which again indicates that the latter cannot be used as a measure of the former.

Since at birth the brain cells are reported to be predominantly neurons (*8*), it is likely that the decrease has indeed affected the number of neurons. Since, as discussed above, the neurons essentially do not divide any more after birth, any neuron deficiency at birth may persist throughout the life of the animal. Such deficiency may contribute to the impaired behavior of the offspring of protein-deficient mothers that has been reported in the literature.

The change in protein content, twice as large as that in DNA, indicates that not only the number of cells was altered but also the cells are qualitatively different. Whether these qualitative changes are irreversible or whether they merely represent a delay in maturation is still not known. However, when evaluated at 3 months of age, the experimental animals manifested abnormalities of gait and response to environmental stimuli.

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Tritiated Digoxin Binding to ($Na^+ + K^+$)-Activated Adenosine Triphosphatase: Possible Allosteric Site

Abstract. *Tritiated H^3-digoxin specifically binds to a cardiac ($Na^+ + K^+$)-activated adenosine triphosphatase. In the presence of adenosine triphosphate and other nucleoside di- and triphosphates, binding is stimulated by sodium ion, the apparent rate constant being similar to that reported for phosphorus-32 incorporation from adenosine triphosphate and for adenosine triphosphatase activity. In the presence of magnesium, manganese, inorganic phosphate, or other ions, sodium ion inhibits binding. The data support an allosteric type of sodium-potassium ion pump.*

A particulate Na^+,K^+-adenosine triphosphatase system apparently forms the enzymatic basis for active coupled-electrolyte transport in a variety of tissues (*1*). One of the important characteristics of the enzyme complex is the specific inhibition of activity produced by active cardiac glycosides, ascribed to a possible binding at a K^+-dependent site (*1*, *2*). However, the involvement of both Na^+ and K^+ in glycoside-induced inhibition and the fact that temperature causes alterations of the enzyme's sensitivity to such drugs indicate the complexity of the digitalis-enzyme interaction (*3*). Isotopically labeled digoxin and ouabain were used to determine the mechanism of action of the glycoside-induced inhibition. Initial studies indicated that the drug was actively bound to, and that it probably "stabilized" the phosphorylated enzyme (*4*). This finding would be of importance in view of the accumulated evidence in favor of a functional phosphorylated intermediate in the mechanism of action of the $Na^+ + K^+$)-activated adenosine triphosphatase (*5*). The data are interpreted as involving a Na^+-stimulated phosphorylation of the enzyme system by adenosine triphosphate (ATP) (*5*) followed by a K^+-dependent phosphate reaction (*6*).

The reaction sequence has been conveniently abbreviated as follows:

$$E + ATP \overset{Na^+}{\rightleftharpoons} E\text{-}P + ADP \quad (1)$$

$$E\text{-}P \overset{K^+}{\rightarrow} E + P_i \quad (2)$$

The compound E-P may be one of a number of phosphorylated intermediates, existing perhaps at different energy levels (*1*, *5*). Cardiac glycosides

may inhibit the Na^+,K^+-dependent ATP hydrolysis by interacting with a phosphorylated intermediate, rendering it relatively insensitive to the action of K^+ (*4*, *7*).

We now report further complexities of the glycoside-enzyme interaction, which indicate that, under certain specific conditions, the binding sites may not necessarily be phosphorylated, a finding which has relevance to the role of the Na^+,K^+-adenosine triphosphatase in ion transport.

H^3-Digoxin (approximately 125 count min^{-1} $pmole^{-1}$) was treated with 0.5 to 1.0 mg of a cardiac (Na^+,K^+)-activated adenosine triphosphatase (specific activity, 26 to 40 μmole of inorganic phosphate per milligram of protein per hour) in the presence of ions, various nucleosides and nucleotides, under various conditions, and in the presence and absence of unlabeled ouabain or digoxin ($10^{-4}M$). Morphologically, the enzyme consists of minute vesicles (*2*). Unlabeled ouabain or digoxin competes with labeled digoxin for the same receptor sites on the enzyme and dilutes the radioactivity to a level attributed to nonspecific adsorption (*4*). The reaction was terminated by rapid centrifugation, and the incorporation of digoxin was determined by assay of the alkali-dissolved pellet (0.3 ml of 0.2*N* NaOH). Specific binding is defined as the radioactivity in the pellet obtained from tubes incubated in the presence of H^3-digoxin, minus the radioactivity measured in the presence of H^3-digoxin plus $10^{-4}M$ unlabeled ouabain or digoxin (*4*). Figure 1 indicates the apparent saturation kinetics of digoxin incorporation, suggesting a specificity for the reaction. As demonstrated (*4*), only active cardiac glycosides inhibit the binding. For example, hexahydroscillaren, unlike ouabain or digoxin in similar concentrations, does not significantly dilute the incorporation of the labeled glycoside. The incorporation system (Fig. 1) contains Mg^{2+}, Na^+, and adenosine triphosphate (ATP), conditions which are also employed for the labeling of the enzyme by ATP^{32} (*5*). Addition of K^+ inhibits or partially dissipates the binding (*4*). Similarly, K^+ catalyzes a dephosphorylation of the "phosphorylated intermediate" (*5*, *6*). From preliminary data, half-maximum concentration of Na^+ required for optimum H^3-digoxin binding appears to be in a range similar to that observed both for ATP^{32} incorporation and for the adenosine triphosphatase activity (*1*, *2*). Calculations can be made (Fig. 1, inset) of the relation between amount of cardiac glycoside bound and ATP hydrolyzed; that is, the ratio between the number of picomoles of inorganic phosphate released and the number of picomoles of H^3-digoxin incorporated per milligram of protein in 1 minute. This value is approximately 17,000 (although there is variation, and in several cases the value is 8500), which is in the same range as the apparent turnover number of the phosphorylated intermediate reported for kidney, heart, brain, and electric eel Na^+,K^+-adenosine triphosphatase (*1*, *5*). The turnover represents the ratio between the amount of ATP hydrolyzed and the number of phosphate groups in E-P. This suggests that cardiac glycoside does in fact interact with a phosphorylated intermediate (in 1 : 1 or 2 : 1 stoichiometry) of the type proposed by numerous investigators (*1*, *5*).

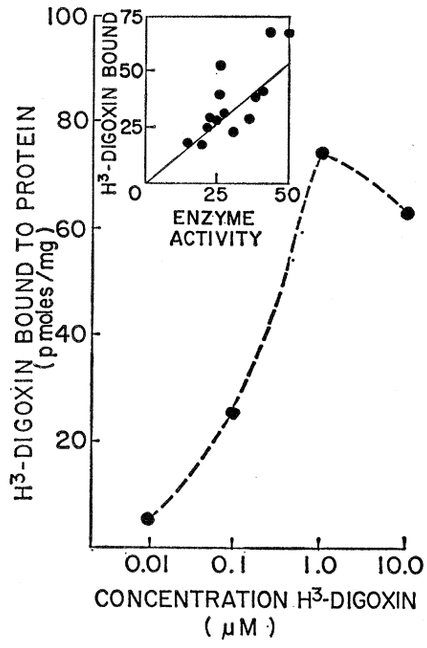

Fig. 1. Concentration and specific binding of H^3-digoxin to a Na^+,K^+-adenosine triphosphatase. The enzyme was prepared from fresh calf heart (*2*) and used within 1 week after isolation. The binding was carried out in tubes containing 50 m*M* tris · HCl (*p*H 7.4), 2 m*M* tris adenosine triphosphate, 5 m*M* $MgCl_2$, 100 m*M* NaCl, 1 m*M* EDTA, H^3-digoxin at indicated concentrations, with and without $10^{-4}M$ unlabeled ouabain; the tubes were shaken in a water bath at 37°C for 3 minutes. The binding reaction is complete in about 1 minute. The inset represents specific binding of $10^{-6}M$ H^3-digoxin as a function of specific activity of various preparations measured as the amount of inorganic phosphate released per milligram of protein per hour in a system identical to the binding experiment, except that KCl (10 m*M*) was added. H^3-digoxin binding is expressed as picomoles per milligram of protein. The hydrolysis of adenosine triphosphate under these conditions is completely inhibited by $10^{-4}M$ ouabain. These results are typical of many experiments.

Digoxin can specifically bind to the enzyme, however, under conditions in which phosphorylated intermediate could not exist, or at best could only be at a minimum. There is a peculiar inhibitory effect of Na^+ on the binding in the absence of nucleotides and in the presence of a variety of ions (Table 1). Apparently, Na^+ stimulates binding only when certain substances are present. In this regard it is of interest that Sr^{2+} supports the Na^+-stimulated type of binding even though E-P would be absent, and Sr^{2+} has only a minimum inhibitory effect on adenosine triphosphatase activity in the concentrations employed. In concentrations which significantly inhibit the enzyme action, Ca^{2+} supports (to a minor degree) the Na^+-inhibited type of binding (Table 1). The fact that adenosine diphosphate (ADP) and cytosine triphosphate (CTP) [also guanosine diphosphate (GDP) and inosine triphosphate (ITP), but the data not shown] or Sr^{2+} behave like ATP strongly suggests that it is not the formation of a phosphorylated enzyme per se that is required for glycoside binding (the above would not be

Table 1. Effect of Na^+ on specific H^3-digoxin binding supported by various cations, nucleoside di- and triphosphates, and anions. The cations, except when otherwise indicated, were employed in 5 m*M* concentration; tris salts of the nucleosides and nucleotides, in 2 m*M* concentration; the anions in 2 to 4 m*M* concentration. The conditions were the same as for Fig. 1, with specific activity of the cardiac enzyme varying between 26 and 40. The results were compiled from many experiments.

Addition	H^3-Digoxin bound to protein (pmole/mg)	
	Without Na^+	100 m*M* with Na^+
None	0	0
ATP	0	10.3
Mg^{2+} + ATP	14.5	37.3
ADP	0.5	0.7
Mg^{2+} + ADP	4.8	11.1
Mg^{2+} + CTP	14.3	26.1
(5 m*M*) Mg^{2+}	11.5	0
(5 m*M*) Mn^{2+}	31.7	16.3
(0.05 m*M*) Ca^{2+}	2.0	0
(0.5 m*M*) Ca^{2+}	2.7	0
(5 m*M*) Ca^{2+}	4.0	0.5
(20 m*M*) Ca^{2+}	9.0	2.9
(5 m*M*) Sr^{2+}	5.3	16.1
(4 m*M*) P_i	0.7	0
P_i + Mg^{2+}	42.8	1.4
Tris-arsenate (As)	0	0
As + Mg^{2+}	38.8	1.9
Tris-SO_4^{2-} (SO_4)	5.4	0
SO_4 + Mg^{2+}	5.6	0
Tris-acetate + Mg^{2+}	13.9	0

expected to form E-P). The specificity of the binding is the same in all cases, since inactive cardiac glycosides do not prevent or dilute the active binding. Even in the absence of added cations, anions, or nucleotides, some specific binding does occur on prolonged incubation. For example, in 3 minutes 1.5 pmole of H^3-digoxin was bound per milligram of protein; in 30 minutes 4.4 pmole, and in 120 minutes 6.2 pmole of H^3-digoxin per milligram of enzyme protein were bound. Na^+,K^+-adenosine triphosphatase activity is not destroyed under these prolonged incubation conditions. This is consistent with studies over protracted incubation periods in a system which continually removes ADP, maintains a constant concentration of ATP, and measures the oxidation of NADH (reduced form of nicotinamide adenine dinucleotide) as an indication of adenosine triphosphatase activity, so that the rate of ATP hydrolysis remains first-order for 1 hour (*8*). Under these conditions, addition of ouabain produces increasing inhibition with time.

These results do not disprove the presence or importance of E-P. They do, however, indicate that the conformational state of the enzyme is probably of primary significance in glycoside binding and hence in the mechanism of its action. The formation of E-P may be one of many ways in which the allosteric nature of the system is altered. Prolonged incubation or interaction with a variety of ions may also change the conformation or "expose" binding sites. The fact that stimulation or inhibition of H^3-digoxin binding by Na^+ can occur under conditions when E-P is maximum, minimum, or absent suggests that the binding site (or sites) becomes more or less accessible depending upon the particular structural state of the vesicle. These complex data are relevant to the allosteric pump concept of Jardetzky (*9*). Sodium ion stimulates hydrolysis of ATP at one site but inhibits at another (the K^+-stimulatory site) (*1, 2*). Incubation of the enzyme with specific substances may effect an "eversion" of the binding sites so that Na^+ now inhibits rather than stimulates glycoside binding. This suggests that digitalis drugs may interact, in intact systems, with both the "phospho" and "dephospho" form of the enzyme transport system (*2, 5*).

As suggested by Skou (*10*), ATP (and presumably other nucleotides) primarily causes an alteration in enzyme structure which somehow leads to the transport of ions. In fact, Skou has stated that E-P may not be of physiological relevance. With reference to phosphoglucomutase, a phosphoenzyme, Koshland has proposed the "induced-fit" or "flexible active site" concept; acyl phosphate formation (which does apparently occur in the Na^+,K^+-adenosine triphosphatase) and subsequent interaction with many cellular constituents may produce specific functional site alterations which are of importance in the activity of the particular enzyme (*11*). As suggested for hormones (*11*), active cardiac glycosides may affect transport systems by functioning as a "feedback-type" of inhibitor, specifically interacting with a regulatory or allosteric site on the transport enzyme system (*4, 12*). The present report supports the concept of an "allosteric type" (*9*) of pump.

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Problem of Sieve-Tube Slime

Abstract. *It is proposed that sieve tubes contain a stationary, living, filamentous reticulum that persists throughout their functioning life. The filaments are about 100 to 150 angstroms in diameter and often striated. By swelling they block the pores of old sieve plates after slime has been lost from the elements. In* Cucurbita, *slime may be loosely bound to the filaments; in many species it is hydrolyzed, swept out by the assimilate stream, and eventually metabolized.*

Recent work with the electron microscope indicates that the pores in the sieve plates of functioning phloem are normally open (*1*). Since open sieve plates should be capable of conducting the assimilate stream by a mass-flow mechanism, one of the principal objections to this theory of translocation has been eliminated. From such work (*1*), in which special methods of preparation avoided opening of the phloem to atmospheric pressure, it becomes evident that most of the impressions of slime- or protoplasm-filled pores were simply artifacts of the methods of preparation.

Probably the most serious problem remaining is the origin, nature, and function of sieve-tube slime. Esau (*2*) states: "The role of slime in this system [the phloem] continues to be an enigma." In reviewing the structure and function of phloem I have come to recognize a situation in phloem structure that seems to explain this enigma.

If one severs the end of a healthy squash stem, phloem exudes rapidly (*3*). Measurements prove that exudation starts with a velocity greater than 1000 cm/hour through the sieve tubes, but, within about 2 minutes, exudation slows and stops. If a 1-mm slice is then cut from the end, exudation resumes, only to stop again; this process may be carried on for hours, and the total volume of exudate collected is many was first hybridized in 0.6*M* NaCl and,