# Finding Cycles in Synchronous Boolean Networks with Applications to Biochemical Systems

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## Abstract

This paper is an analytical study of Boolean networks. The motivation is our desire to understand the large, complicated, and interconnected pathways which comprise intracellular biochemical signal transduction networks. The simplest possible conceptual model that mimics signal transduction with sigmoidal kinetics is the n-node Boolean network each of whose elements or nodes has the value 0 (off) or 1 (on) at any given time  $T = 0, 1, 2, \dots$  A Boolean network has  $2^n$  states all of which are either on periodic cycles (including fixed points) or transients leading to cycles. Thus one understands a Boolean network by determining the number and length of its cycles. The problem one must circumvent is the large number of states  $(2^n)$  since the networks we are interested in have 100 or more elements. Thus we concentrate on developing size n methods rather than the impossible task of enumerating all 2<sup>n</sup> states. This is done as follows: the dynamics of the network can be described by n polynomial equations which describe the logical function which determines the interaction at each node. Iterating the equations one step at a time finds all fixed points, period two cycles, period three cycles, etc. This is a general method that can be used to determine the fixed points and moderately large periodic cycles of any size network, but it is not useful in finding the largest cycles in a large network. However, we also show that the network equations can often be reduced to scalar form, which makes the cycle structure much more transparent. The scalar equations method is a true "size n" method and several examples (including non-trivial biochemical systems) are examined.

## Introduction

We are engaged in a long-term research project to describe and analyze the complicated and interconnected pathways that comprise intracellular biochemical signal transduction networks. These biochemical networks are the mechanism by which signals received via specific receptors on the outer membrane of an individual cell are transduced through the cytoplasm to the cell nucleus [Lodish et al., 1999]. Because signal transduction networks are made up of hundreds of interacting proteins in the membrane, cytoskeleton, cytoplasm, and nucleus, it is difficult to model them with differential equations since many non-linear terms would be required. Additionally, the differential equations approach to modeling requires the use of biochemical parameter information such as initial concentrations of reactants and kinetic parameters of enzymes that have been determined by *in vitro* experimentation. The relevance of parameters derived *in vitro* is not clear as there is much evidence that signaling systems are often restricted in the cell with respect to both location and accessibility [Bray, 1998], a condition that is not present *in vitro*.

We have attempted to avoid these problems by employing the simplest possible conceptual model that bears a resemblance to the networks we wish to study; namely a synchronous Boolean network. A Boolean network is a directed graph—a system of nodes that interact with each other in a specified manner. Interactions between nodes (including the direction of the interaction) are represented by the edges of the graph. In our case, the nodes represent individual signal transduction pathway proteins and the edges indicate which proteins have the potential to interact directly (e.g. phosphorylation). At each successive (discrete) time point, the nodes can exist in one of two different states; 0 (off) or 1 (on). Which of these two states the node will occupy at any given time point is determined by the logic function that is necessarily associated with each node. The logic function for a node is an instruction set for determining the state of a node based on the state(s) of the input node(s) at the previous time point.

Despite the fact of their conceptual simplicity, Boolean networks have applications in many areas including circuit theory and computer science [Dunne, 1988; Tocci & Widmer, 2001]. In molecular biological systems the concepts of Boolean networks have been applied in areas such as the modeling of gene regulation [Jacob, 1961; Kauffman, 1969; Kauffman, 1993], but little has been done in terms of modeling protein-protein interaction. However, with the relatively recent breakthroughs in our understanding of the mechanisms of protein regulation (e.g. phosphorylation, methylation, etc.), it appears that protein activity might be realistically represented by Boolean logic [Volkman *et al.*, 2001]. This is especially true for Michaelis-Menton and Hill kinetics where reaction rates are characterized by sigmoidal (or S-shaped) curves. Steep sigmoidal curves are well approximated by switch-like on-off interactions.

Although the simplifications inherent in them make Boolean networks very attractive for modeling complicated systems, a natural question arises as to how well a Boolean network can represent a real biochemical system given that it does transform the nature of the interactions. Glass [1985] and Glass and Kauffman [1972, 1973] investigated this question and determined that Boolean networks provide an accurate general description of biochemical networks with sigmoidal interactions provided a negative feedback loop (i.e. a feedback loop with an odd number of inhibitory

interactions) is present in the system. Their general principle can be stated as follows [Glass, 1985]: If the associated Boolean network has a cyclic attractor (i.e. a cycle), then one of the following holds: (1) the biochemical network has a stable limit cycle, or (2) the biochemical network has a stable equilibrium. A mathematical formulation and proof of this principle has been given by Glass and Pasternack [1978] for piece-wise linear systems.

More generally we can say that a biochemical network with a negative feedback loop may have periodic cycles if its associated Boolean model network does. If the Boolean model has only fixed points, then its associated biochemical network has no cycles but only stable equilibrium points. The Glass-Kauffman hypothesis is illustrated in detail in Sec. 4. It should be pointed out that there are many known examples of oscillations in biochemical systems, with both positive and negative feedback [Goldbeter, 1996].

Because the biochemical networks we wish to study have negative feedback loops and the Boolean models of those networks we have experimented with so far have the potential for periodic cycles, we are faced with the need to determine when Boolean networks do or do not have cycles. Our approach to a method for determining the existence of periodic cycles in Boolean networks involves two different analytical techniques, both well-known in the theory of dynamical systems and differential equations. The first is iteration of the Boolean logic functions. This technique is highly developed in the use of discrete dynamical systems or maps [Alligood *et al.*, 1997]. It is the most general technique that can be useful for finding moderately size cycles in any network. The second technique, reduction to a scalar equation, is a time-honored method in differential equations (used recently by one of the authors [Zhang & Heidel, 1997; Heidel & Zhang, 1999]). It does not always yield results, but it is very powerful when it does.

## 2. Analytical Analysis of Boolean Networks

A Boolean network is a set of n nodes, each of which is either in state 1 (on) or state 0 (off) at any given time T. Each node is then updated at time T+1 by inputs from any fixed set of k other nodes according to any desired logical rule. Since each of the n states can be sequentially either off or on there is a total of  $2^n$  different possible set of states for the entire network.

Since the total number of states is finite and the network changes states sequentially in discrete time steps, the network must necessarily return to a previously occupied state. With n nodes, the largest possible number of time points before reencountering a previous state is  $2^n$ . This means that all possible trajectories of the network consist of either cycles (loops) of any length from size one (a fixed point) to a maximum of  $2^n$ , or transient states leading to a cycle.

A complete description of a Boolean network consists of (i) the nodes with interconnecting arrows indicating which nodes affect each other (a directed graph), (ii) a logical table indicating the complete logic which controls each node, and (iii) a diagram showing the interconnections (i.e. cycles and transient states) between all  $2^n$  states.

Of course this is an ideal total description which can only be realized for small n since one would have to account for  $2^n$  states. That would be unfeasible even for an n = 10 network since there would be 1024 possible states.

Thus methods are desired that provide an adequate description of the network utilizing quantities of size n rather than size  $2^n$  without having to completely enumerate all states. A first step has been taken in this direction [Cull, 1971; Robert, 1986]. The logic describing the update for each node can be described by a polynomial function in n variables with coefficients of 0 and 1 using Boolean algebra. These n functions, one for each node, encode all of the information about the system. This is most easily understood by looking at some simple examples, such as the one shown in Fig. 1. The network shown in Panel A has three nodes, all of which are connected to each other. The logic of the connections is shown in Panel B, all of which are OR. The complete logical description and state space structures  $(2^3 = 8)$  are easily determined and shown in Panel D, while the map of the trajectories is shown in Panel C.

The complete system description shown in Fig. 1 can also be obtained with logical function analysis. It is easily verified that the logic for the three nodes is given by:

$$f_A = B + C + BC \tag{1}$$

$$f_{B} = A + C + AC$$
(2)  
$$f_{C} = A + B + AB$$
(3)

$$S_{\rm C} = A + B + AB \tag{3}$$

Fixed points (cycles of length one) can then be determined by setting each logical function equal to the current state as follows:

$$A = f_A = B + C + BC \tag{4}$$

$$C = f_C = A + B + AB \tag{6}$$

which can easily be solved to obtain A = B = C under the rules of Boolean algebra (standard arithmetic, mod 2). Thus the fixed points are (0,0,0) and (1,1,1).

In a similar manner, antecedents (states that lead immediately to the fixed points) can be determined by setting the logic functions equal to each fixed point:

$$0 = f_A = B + C + BC \tag{7}$$

$$0 = f_B = A + C + AC$$

$$0 = f_C = A + B + AB$$
(8)
(9)

$$\mathbf{f}_{\mathrm{C}} = \mathbf{A} + \mathbf{B} + \mathbf{A}\mathbf{B} \tag{9}$$

and

1

$$1 = f_A = B + C + BC \tag{10}$$

$$= f_{\rm B} = \mathbf{A} + \mathbf{C} + \mathbf{A}\mathbf{C} \tag{11}$$

$$I = f_C = A + B + AB \tag{12}$$

The first system cannot be solved (if A = 1, then 0 = 1 + C + C = 1, a contradiction), so there are no antecedents to the fixed point (0,0,0), as is verified in Panel C of Fig. 1. The second system of equations is solvable and yields the points (0,1,1), (1,0,1), (1,1,0), and (1,1,1) as the antecedents of (1,1,1). The antecedents of the first three antecedents (found by the same method) are (1,0,0), (0,1,0), and (0,0,1), respectively. Thus, working with the three functions f<sub>A</sub>, f<sub>B</sub>, and f<sub>C</sub> (which, of course, are determined directly from the three

elementary logic tables) the complete state space structure is determined.

Another simple network is shown in Fig. 2. Unlike the first network, this network contains two periodic orbits and no fixed points. The logic functions for this network are:

$$\mathbf{f}_{\mathrm{A}} = \mathbf{1} + \mathbf{C} \tag{13}$$

$$\mathbf{f}_{\mathrm{B}} = \mathbf{A} \tag{14}$$

$$f_{\rm C} = B \tag{15}$$

To determine the fixed points we set

$$\mathbf{A} = \mathbf{f}_{\mathbf{A}} = \mathbf{1} + \mathbf{C} \tag{16}$$

$$\mathbf{B} = \mathbf{f}_{\mathbf{B}} = \mathbf{A} \tag{17}$$

$$C = f_C = B \tag{18}$$

as before. This system is clearly not solvable, so there are no fixed points. Finding periodic points is achieved by iteration:

$$A = f_A^2 = 1 + B$$
(19)  
$$B = f^2 = 1 + C$$
(20)

$$B = t_B = 1 + C$$
(20)

$$C = f_C^2 = A \tag{21}$$

This system has the solutions (1,0,1) and (0,1,0) which is the period two cycle shown in Panel C of Fig. 2.

Iteration of the logic functions three and five times (to find period three and period five points, respectively) yields systems that are not solvable so it can be correctly concluded that there are no states of those types. The fourth iteration generates a system identical to the one solved for period two points above. Because period two points are also (trivially) period four points, this result means that there are no true period four points. Similarly, six iterations yields a system of equations

$$A = f_A^{\ 6} = A$$
(22)  
$$B = f_B^{\ 6} = B$$
(23)

$$= B \tag{23}$$

$$C = f_C^{6} = 1 + 1 + C = C$$
(24)

which is true for every one of the eight points in the state space of this network. Since two of the points have been determined to be period two points (which are also trivially period six points), the remaining six points in the state space must comprise a true period six orbit, which is verified to be true in Fig. 2.

The above algebraic approach using the n node logic functions can be carried out on quite large systems when searching for moderately sized cycles. However, finding all cycles in a large system could involve an enormous number of iterative computations (if very long cycles are present) meaning that the largest cycles of a large system might not be found using this method.

#### **3.** The Scalar Equation Method

The method of iteration for finding cycles, described in the last section, is

completely general which means that this method is impractical to use for finding all of the cycles in large systems. Even with the aid of a computer, the largest system for which it would be possible to determine all potential cycles would be approximately n = 25 (which has the potential for a cycle of size  $2^{25} = 33,554,432$ ). However, sometimes the number of equations necessary to describe the logic of a given system can be reduced to a smaller set of higher order equations, perhaps only a single equation. Such a scalar equation is more transparent to analyze for cycles.

Looking again at the network described in Fig. 2, it can be seen that the logic functions that were used previously can be converted to the following time-dependent logic equations

$$\begin{array}{ll} A_{T+1} = \ 1 + C_T & (25) \\ B_{T+1} = \ A_T & (26) \\ C_{T+1} = \ B_T & (27) \end{array}$$

From these equations, it can be easily verified that  $A_{T+3} = 1 + A_T$  (with similar equations for  $B_T$  and  $C_T$ ). From this simple scalar equation it follows immediately that all elements of the 8-dimensional state space lie on an orbit of period six. It also follows immediately from this equation that there are no fixed points and no period three cycles. Thus the only possibility besides a full period six cycle is a period two cycle. We have already seen (and it is easily verified) that a period two cycle exists. Thus the entire state space consists of a period two cycle and a period six cycle.

The network in Fig. 2 is a simple case of an affine system (linear terms plus constant terms). Affine Boolean networks have been studied in great detail and their cyclic structure is completely understood in a general way [Wilson & Milligan, 1992; Milligan & Wilson, 1993]. However, since affine systems are a very restricted class of Boolean networks and will only rarely arise in applications, we do not make use of the highly developed theory of affine systems. Rather we use an occasional example of a linear system in illustrating our very general techniques. The network shown in Fig. 3 is one such example; it is an extension of the previous example as it contains six nodes and analogous logic (shown in Panel B). The logic for this system can be expressed by the following logic functions:

$$A_{T+1} = 1 + F_T$$
 (28)

$$\mathbf{B}_{\mathrm{T}+1} = \mathbf{A}_{\mathrm{T}} \tag{29}$$

$$C_{T+1} = B_T \tag{30}$$

$$D_{T+1} = C_T$$
 (31)  
 $E_{T+1} = D_T$  (32)

$$F_{T+1} = F_T$$
 (32)

Proceeding as above, we easily find that  $A_{T+6} = 1 + A_T$ . This means that all states lie on cycles of period 12 and, furthermore, there are no cycles of period six. Thus there are no cycles of period one (fixed points), two, or three since these would also have period six. Thus all cycles have either period four or period 12. Since  $2^6 = 64 = 5$  (12 + 4, there are five distinct cycles of period 12 and one cycle of period four.

Clearly linear examples such as these can be extended to arbitrarily large size and

any single loop whose connections have simple off or on logic can be analyzed in a similar way. We now turn our attention to nonlinear logical functions for which there are few general procedures and show that the scalar approach continues to be very useful.

The network shown in Fig. 4 has the logical equation description:

$$A_{T+1} = B_T D_T \tag{34}$$

$$B_{T+1} = 1 + A_T$$
 (35)  
 $C_{T+1} = B_T$  (36)

$$D_{T+1} = C_T.$$
 (30)

$$D_{T+1} = C_T. \tag{3}$$

We readily compute that  $A_{T+4} = (1 + A_{T+2})(1 + A_T)$ . Thus the scalar equation separates into two parts where the state of A at T + 4 depends on the state of A at both T and T + 2. That means that the state of A at even time points is dependent only on the previous two even time points, while the state of A at odd time points is dependent on the two previous odd time points. Thus the state of A at the first two even and odd time points must be specified and then the system can move along the trajectory mandated by the logic. For example, consider the even time steps where the values at T = 0, 2 are specified and later values are determined from the scalar equation. The possible trajectories at each even time point is given by the following tables:

T =	0 2	4	2 4	6	4 6	8	68	10	8	10	12
	0 0	1	0 1	0	1 0	0	0 0	1	0	1	0
	0 1	0	1 0	0	0 0	1	0 1	0	1	0	0
	1 0	0	0 0	1	0 1	0	1 0	0	0	0	1
	1 1	0	1 0	0	$0 \ 0$	1	0 1	0	1	0	0

Regardless of how the system starts, it moves to a period 3 orbit on the even time points. Using the same method, it can be shown that the system moves to the same period three orbit on the odd time points (not shown). When both even and odd times are considered together, there are only two different orbits that are obtained for node A; (0,0,1) or (0,0,0,0,1,1). That is, the value of node A is in either a period three or period six orbit. Relating  $B_{T+1}$ ,  $C_{T+1}$ , and  $D_{T+1}$  to  $A_T$  using the logic equations, we obtain either a period three cycle :

$$(0,1,0,1) \rightarrow (1,1,1,0) \rightarrow (0,0,1,1,) \rightarrow (0,1,0,1)$$

or a period six cycle:

$$(0,1,0,0) \rightarrow (0,1,1,0) \rightarrow (0,1,1,1) \rightarrow (1,1,1,1) \rightarrow (1,0,1,1) \rightarrow (0,0,0,1) \rightarrow (0,1,0,0)$$

As is typical for nonlinear logics there are also seven  $(3 + 6 + 7 = 16 = 2^4)$  transient states which lead to one or the other of the periodic cycles.

Another example of a nonlinear network is shown in Fig. 5. The logic functions for this network are:

$$A_{T+1} = B_T C_T \tag{38}$$

$$B_{T+1} = 1 + A_T$$
(39)  

$$C_{T+1} = B_T$$
(40)

$$C_{T+1} = B_T \tag{40}$$

This network would immediately appear to be simpler than the proceeding example and, in fact, it has only one cycle instead of two. Surprisingly, however, one aspect of the analysis is more complicated than for the previous four node system. Determining the scalar equations we find that

$$A_{T+3} = (1 + A_{T+1})(1 + A_T)$$
(41)

with similar equations for  $B_T$  and  $C_T$ . In order to determine all of the sequential possibilities for the state of node A in this equation we have to specify the first three elements of the sequence, then use the first two to determine the third, the second two to determine the fourth, etc., as follows:

T =	0 1 2	3	0 1	3	1 2	4	2 3	5	3	4	6
	0 0 0	1	0 0	1	0 0	1	0 0	1	0	0	1
	0 0 1	1	0 1	0	0 1	0	0 1	0	0	1	0
	$0 \ 1 \ 0$	0	1 0	0	1 0	0	1 0	0	1	0	0
	0 1 1	0	1 1	0	1 1	0	1 1	0	1	1	0
	$1 \ 0 \ 0$	0		I	•		ľ				
	1 0 1	0									
	1 1 0	0									
	1 1 1	0									

Thus there is a single period five orbit (0,0,0,1,1) for node A and the period five cycle

$$(0,0,0) \rightarrow (0,1,0) \rightarrow (0,1,1) \rightarrow (1,1,1) \rightarrow (1,0,1) \rightarrow (0,0,0)$$

for the full system with three transients  $(3 + 5 = 8 = 2^3)$ .

The next example, shown in Fig. 6, is from Robert [1986]. This network has the logical functions:

$$A_{T+1} = B_T + B_T C_T$$
(42)  

$$B_{T+1} = A_T + A_T C_T$$
(43)  

$$C_{T+1} = 1 + A_T + B_T + A_T B_T$$
(44)

It is easily shown that  $A_{T+3} = A_{T+1}$  with similar equations for  $B_T$  and  $C_T$ . This equation again alternates between even and odd time intervals. For example, we have

T = 0	2	2	4	4	6
0	0	0	0	0	0
1	1	1	1	1	1

with an identical set of sequences for the odd time intervals. This clearly gives the fixed points (1,1,0), (0,0,1) and the period two orbit:

$$(0,1,0) \rightarrow (1,0,0) \rightarrow (0,1,0)$$

with four transient states.

The next examples illustrate how Boolean networks can be used to model a molecular biological system. The first is the epigenetic model considered by Goodwin [1963] and is shown in Fig. 7. It is derived directly from idealized protein-nucleic acid interactions involved in gene regulation. The logic functions are easily seen to be:

$$L_{1(T+1)} = 1 + C_{1(T)} + C_{2(T)} + C_{1(T)}C_{2(T)}$$
(45)

$$\begin{array}{c} R_{1(T+1)} = L_{1(T)} \\ C &= P \\ \end{array} \tag{46}$$

$$C_{1(T+1)} = R_{1(T)}$$
 (47)

$$L_{2(T+1)} = 1 + C_{1(T)} + C_{2(T)} + C_{1(T)}C_{2(T)}$$
(48)

$$R_{2(T+1)} = L_{2(T)}$$
(49)  

$$C_{2(T+1)} = R_{2(T)}$$
(50)

$$C_{2(T+1)} = R_{2(T)}$$
 (2)

We see that  $L_{1(T+1)} = L_{2(T+1)}$  and it is easily derived that  $L_{1(T+3)} = 1 + L_{1(T)} + L_{2(T)} + L_{2(T)}$  $L_{1(T)}L_{2(T)}$ . Thus  $L_{1(T+4)} = 1 + L_{1(T+1)}$ . This equation tells us that any cycle has period six, but is not period three. Thus there are no fixed points but there could be a period two cycle. Performing the familiar iteration procedure described in Sec. 2 produces the period two cycle  $(1,0,1,1,0,1) \rightarrow (0,1,0,0,1,0) \rightarrow (1,0,1,1,0,1)$ . Using the equation  $L_{1(T+4)} = 1 + 1$  $L_{1(T+1)}$  produces a single period six cycle  $(1,1,1,1,1,1) \rightarrow \dots$ . Obviously this leaves 64-2-6 = 56 transient states.

A more complicated epigenetic system is shown in Fig. 8 [Goodwin, 1963]. This system was created by expanding the original system by adding another unit of three components. The logic functions are given by:

$$L_{1(T+1)} = 1 + C_{1(T)} + C_{2(T)} + C_{1(T)}C_{2(T)}$$
(51)

$$R_{1(T+1)} = L_{1(T)}$$

$$C_{1(T+1)} = R_{1(T)}$$

$$L_{2(T+1)} = 1 + C_{1(T)} + C_{2(T)} + C_{1(T)}C_{2(T)} + C_{1(T)}C_{3(T)} + C_{2(T)}C_{3(T)} + C_{1(T)}C_{2(T)}C_{3(T)}$$
(54)

$$R_{2(T+1)} = L_{2(T)}$$

$$C_{2(T+1)} = R_{2(T)}$$
(54)

$$L_{3(T+1)} = 1 + C_{2(T)} + C_{3(T)} + C_{2(T)}C_{3(T)}$$
(57)  

$$R_{3(T+1)} = L_{3(T)}$$
(58)  

$$C_{3(T+1)} = R_{3(T)}$$
(59)

Proceeding as in the previous example we obtain the three equations:

$$L_{1(T+3)} = (1 + L_{1(T)})(1 + L_{2(T)})$$
(60)

$$L_{2(T+3)} = L_{1(T+3)}L_{3(T+3)}$$
(61)

$$L_{3(T+3)} = (1 + L_{2(T)})(1 + L_{3(T)})$$
(62)

which means we can then write:

$$L_{1(T+6)} = (1 + L_{1(T+3)})(1 + L_{1(T+3)}L_{3(T+3)})$$
(63)

$$= 1 + L_{1(T+3)}L_{3(T+3)} + L_{1(T+3)} + L_{1(T+3)}L_{3(T+3)}$$

$$= 1 + L_{1(T+3)}$$
(64)
(65)

Likewise  $L_{3(T+6)} = 1 + L_{3(T+3)}$ , thus  $L_1$  and  $L_3$  both (independently) have period six cycles which are not period three cycles. Thus there are no fixed points, but checking for period cvcles iteration, we find two by two cycles  $(0,1,0,1,1,1,1,0,1) \rightarrow (1,0,1,0,0,0,0,1,0).$ 

The period six cycle(s) for  $L_1$  (or  $L_3$ ) can be found from the table:

T = 0	0	0	0	0	1	1	1	1
T = 1	0	0	1	1	0	0	1	1
T = 2	0	1	0	1	0	1	0	1
T = 3	1	1	1	1	0	0	0	0
T = 4	1	1	0	0	1	1	0	0
T = 5	1	0	1	0	1	0	1	0

Thus  $L_1$  and  $L_3$  each (separately) have just one period six cycle. But since they are independent there is then a total of six different period six cycles for the full nine node network. Clearly this triple chain can be extended to a chain of arbitrary length [Goodwin, 1963] and analyzed in a similar manner as above becoming more complicated at each step.

Another idealized example, shown in Fig. 9, was considered by Gonze and Goldbetter [2000] as a model of phosphorylation/dephosphorylation cycles. Unlike the last example, this one is a model of protein-protein interactions; the type most related to signal transduction. From the logic tables were derived the following logic functions:

$$\mathbf{A}_{\mathrm{T}+1} = \mathbf{B}_{\mathrm{T}} \tag{66}$$

$$B_{T+1} = C_T$$
 (67)  
 $C_{T+1} = D_T$  (68)

$$T_{T+1} = D_T \tag{68}$$

$$D_{T+1} = A_T \tag{69}$$

that reduce to the scalar equations

$$A_{T+4} = A_T \tag{70}$$

$$\mathbf{B}_{\mathrm{T}+4} = \mathbf{B}_{\mathrm{T}} \tag{71}$$

$$C_{T+4} = C_T \tag{72}$$

$$D_{T+4} = D_T \tag{73}$$

which means that there are three period four cycles, one period two cycle, and two fixed points in the system.

From the above examples it is clear that a wide variety of Boolean networks have periodic cycles in their dynamics. In fact networks that have any desired cycle structure can be readily conceived. However, we have found that very small single changes in logic or structure can have drastic effects on the existence of cycles in the state space of the network. For example, simply changing the logic for node B in Fig. 5 from ON to OFF results in the system moving only to the fixed points (0,0,0) and (1,1,1). Similarly, when the logic for node B in Fig. 4 is changed from ON to OFF, there is only one fixed point; (0,0,0,0). Thus, adding a node or connection to a network with periodic cycles tends to eliminate the cycles. This observation may have significance for modeling biological mutations.

Reducing the logical functions to a single scalar equation is clearly a simple and effective method of analyzing the trajectories of Boolean networks. More realistic applications of this technique to biochemical networks are give in a later section.

## 4. The Glass-Kauffman Hypothesis.

Both the protein-nucleic acid model (Figs. 7 and 8) and the protein-protein model (Fig. 9) were originally analyzed with differential equations. In the case of Fig. 9 the differential equation model, with steep sigmoid kinetics, leads to a limit cycle. Similarly, our Boolean model demonstrates periodic cycles. This raises the question as to the meaning of cycles in a Boolean model—specifically, does the presence of cycles or fixed points in the Boolean model indicate limit cycles or equilibrium points in the differential equations model. As stated earlier, the Glass-Kauffman hypothesis indicates that in many cases Boolean cycle structure can give information regarding the analog dynamics. Assuming the system modeled has negative feedback (i.e., an odd number of negative interactions in a loop), the hypothesis can be summarized as having two main components; (i) when the Boolean model has no cycles and (ii) when the Boolean model has cycles. In the first case the hypothesis states that the differential equation model of the system will not have limit cycles, while in the second case the differential equation model may or may not have cycles. Thus, the Glass-Kauffman hypothesis answers the original question by saying that the cycle structure of a Boolean model can be used to rule in or out the possibility of cycles in a differential equation system.

In formulating their hypothesis, Glass and Kauffman [1972, 1973] emphasize that it is an intuitive guide and especially that the analogy does not depend on the exact nature (i.e., steepness) of the sigmoidal kinetics. In fact it is only well-understood for piece-wise continuous (switching function) differential equations [Glass and Pasternack, 1978]. In these papers all of the examples given are for the single case where both the differential equation systems and their Boolean analogues have cycles. In this section we discuss three similar examples of sigmoidal kinetics which illustrate the three different possibilities of the Glass-Kauffman hypothesis. Note that in all three cases the requisite negative feedback is present.

The first case to be considered is when the Boolean model does not have cycles. This is the strongest case since it suggests that the analog model will not have cycles. The system is represented with the differential equations

$$\frac{\mathrm{dA}}{\mathrm{dt}} = \frac{\mathrm{B}^2}{1+\mathrm{B}^2} \cdot \frac{\mathrm{D}^2}{1+\mathrm{D}^2} - \mathrm{A}$$
(74)

$$\frac{\mathrm{dB}}{\mathrm{dt}} = \frac{\mathrm{A}^2}{1+\mathrm{A}^2} - \mathrm{B}$$
(75)

$$\frac{\mathrm{dC}}{\mathrm{dt}} = \frac{\mathrm{B}^2}{1+\mathrm{B}^2} - \mathrm{C}$$
(76)

$$\frac{\mathrm{d}\mathrm{D}}{\mathrm{d}\mathrm{t}} = \frac{1}{1+\mathrm{C}^2} - \mathrm{D} \tag{77}$$

which is easily shown numerically to have the stable equilibrium A = B = C = 0, D = 1. The Boolean model of this system (which is identical to the model in Fig. 4, but with the negative logic moved to the C/D connection) has the logical equations

$$A_{T+1} = B_T D_T \tag{78}$$

$$B_{T+1} = A_T$$
 (79)  
 $C_{T+1} = B_T$  (80)

$$D_{T+1} = 1 + C_T$$
(81)

It is easily shown that the scalar equation is  $A_{T+4} = A_{T+2}(1 + A_T)$  which leads only to the fixed point (0,0,0,1). Thus, this is an example of the hypothesis (extended to sigmoidal kinetics) that no cycles in the Boolean model implies no cycles in the differential equation model.

The second case to be considered is when the Boolean model does exhibit cycles. We will first look at an example when the differential equations analogue does contain cycles. In that example, due to Glass and Pasternack [1978b], the four dimensional system

$$\frac{dA}{dt} = \frac{0.0625}{0.0625 + D^4} - A$$
(82)

$$\frac{dB}{dt} = \frac{0.0625A^4}{0.0625 + A^4} - B$$
(82)

$$\frac{dC}{dt} = \frac{0.0625B^4}{0.0625 + B^4} - C$$
(83)

$$\frac{dD}{dt} = \frac{0.0625C^4}{0.0625 + C^4} - D$$
(84)

has a limit cycle. The analogous Boolean network, shown in Fig. 10, has the logical equations

$$A_{T+1} = 1 + D_T$$
 (85)

$$B_{T+1} = A_T \tag{86}$$

$$C_{T+1} = B_T \tag{87}$$

$$C_{T+1} = B_T \tag{87}$$

$$D_{T+1} = C_T \tag{88}$$

Direct computation shows that  $A_{T+4} = 1 + A_T$  with identical equations for nodes B, C, and D. Thus all cycles have period 8 and there are no fixed points, period two cycles, or period four cycles. In fact there are two period 8 cycles. Thus the system of differential equations and its Boolean analogue both have cycles.

The final example illustrates the second possibility when the Boolean model exhibits cycles—that the differential equations system may go only to equilibrium points. Such a system is described by the equations

$$\frac{\mathrm{dA}}{\mathrm{dt}} = \frac{\mathrm{B}^2}{1+\mathrm{B}^2} \cdot \frac{\mathrm{D}^2}{1+\mathrm{D}^2} - \mathrm{A} \tag{89}$$

$$\frac{\mathrm{dB}}{\mathrm{dt}} = \frac{1}{1+\mathrm{A}^2} - \mathrm{B}$$
(90)

$$\frac{\mathrm{dC}}{\mathrm{dt}} = \frac{\mathrm{B}^2}{1+\mathrm{B}^2} - \mathrm{C}$$
(91)

$$\frac{\mathrm{d}\mathrm{D}}{\mathrm{d}\mathrm{t}} = \frac{\mathrm{C}^2}{1+\mathrm{C}^2} - \mathrm{D}$$
(92)

and it is easily shown numerically to have the single stable equilibrium (A = 0.02, B = 1, C = 0.5, D = 0.2). The Boolean analogue (already shown in Fig. 4) has periodic cycles (period 3 and 6). Thus the system of differential equations may fail to have a limit cycle while its Boolean analogue does have one or more cycles.

The last possibility is the least desirable as the Boolean model does not mimic the analog behavior. Therefore, a major improvement in the Glass-Kauffman hypothesis would be a method to determine more completely what types of logical systems are associated with this case. In any event, the hypothesis is useful in its ability to rule out the possibility of cycles under certain conditions.

#### 5. Biochemical Networks

In this section we apply our techniques for finding cycles in Boolean networks to two examples in the literature. Rather than the purely illustrative examples of the previous sections, these examples are serious attempts to model real biological networks. In both cases, the cycle structure was solved by the scalar equation method.

The first example is coupled oscillations in the cell cycle. Research into the mechanism of cell cycle regulation makes it apparent that the onset of M (mitosis) and S (DNA replication) phases of the cell cycle are controlled by the periodic activation of cyclin-dependent kinases (cdk's) [Stillman, 1996]. A differential equations model of the system was created [Romond, *et al.*, 1999] from which we derived the logic and created the Boolean model shown in Fig. 11. This time the logic functions are:

$$C_{1(T+1)} = 1 + X_{1(T)} + M_{2(T)} + X_{1(T)}M_{2(T)}$$
(93)

 $M_{1(T+1)} = C_{1(T)}$  $X_{1(T+1)} = M_{1(T+1)}$ (94)

$$A_{1}(T+1) - M_{1}(T)$$
 (95)

$$C_{2(T+1)} = I + X_{2(T)} + M_{1(T)} + X_{2(T)}M_{1(T)}$$

$$M_{1(T)} = C$$
(96)

$$M_{2(T+1)} = C_{2(T)}$$
(97)  

$$X_{2(T+1)} = M_{2(T)}$$
(98)

$$_{2(T+1)} = M_{2(T)}$$

From the structure of the logic functions it is natural to try to express the six dimensional system in terms of higher order equations involving just  $C_1$  and  $C_2$ . This gives

$$C_{1(T+3)} = (1 + C_{1(T)})(1 + C_{2(T+1)})$$
(99)  

$$C_{2(T+3)} = (1 + C_{2(T)})(1 + C_{1(T+1)})$$
(100)

For this example it does not appear possible to find a single higher order equation in just one variable. Thus we must work with the two scalar equations at once. Since the values of  $C_1$  and  $C_2$  at time T = 3 are determined by their values at time T = 0 and T = 1, to determine all possible sequences of corresponding C1 and C2 values it is necessary to consider all possible combinations of specifying  $C_1$  at times T = 0, 1, 2 and likewise  $C_2$  at times T = 0, 1, 2. This means that for each of the possible sequences for  $C_{1(0)}$ ,  $C_{1(1)}$ , and  $C_{1(2)}$ , there are eight possible sequences of  $C_{2(0)}$ ,  $C_{2(1)}$ , and  $C_{2(2)}$  for a total possible set of sequences 8 x 8 =  $64 = 2^6$ . Each of these eight sets of eight sequences produces the same periodic orbits of period 2, 5, or 10 (there are no fixed points). Because of the complexity of this analysis we list one of these eight sets:

				$C_1$									$C_2$			
T = 0				0					0	0	0	0	1	1	1	1
T = 1				0					0	0	1	1	0	0	1	1
<u>T = 2</u>				0					0	1	0	1	0	1	0	1
T = 3	1	1	0	0	1	1	0	0	1	1	1	1	0	0	0	0
T = 4	1	0	1	0	1	0	1	0	1	1	0	0	1	1	0	0
T = 5	0	0	0	0	1	1	1	1	0	0	1	0	0	0	1	0
T = 6	0	0	1	1	0	0	1	1	0	0	0	0	0	1	0	1
T = 7	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1
T = 8	1	1	1	1	0	0	0	0	1	1	0	0	1	1	0	0
T = 9	1	1	0	0	1	1	0	0	1	0	1	0	1	0	1	0
Period	5	10	2	10	10	2	10	5	5	10	2	10	10	2	10	5

Combining this chart with the logic equations it is easy to compute that the period two orbit is given by:

$C_1$	$M_1$	$X_1$	$C_2$	$M_2$	$X_2$
(1	0	1	0	1	0)
(0	1	0	1	0	1),

the period five orbit by the five states:

$C_1$	$M_1$	$X_1$	$C_2$	$M_2$	$X_2$
(1	0	0	1	0	0)
(1	1	0	1	1	0)
(0	1	1	0	1	1)
(0	0	1	0	0	1)
(0	0	0	0	0	0),

while the period ten orbit is comprised of the states:

$C_1$	$M_1$	$X_1$	$C_2$	$M_2$	$X_2$
(1	0	0	1	1	0)
(0	1	0	1	1	1)
(0	0	1	0	1	1)
(0	0	0	0	0	1)
(1	0	0	0	0	0)
(1	1	0	1	0	0)
(1	1	1	0	1	0)
(0	1	1	0	0	1)
(0	0	1	0	0	0)
(0	0	0	1	0	0).

Analyzed with differential equations using sigmoidal kinetics, this system is chaotic for certain parameter ranges, an observation that is consistent with the more complicated scalar structure of the logic equation (a pair of equations is required instead of just one). Additionally, for all of the periodic cycles of the Boolean model of this system, each element of the system changes values with some periodicity. In other words, any cycle the system eventually settles into has no elements that are "frozen" in an on or off state. The existence of attractors that do not contain a frozen core of elements has been hypothesized to be another characteristic of Boolean systems with the potential for chaos [Kauffman, 1993]. Finally, when we determined numerically the basins of attraction for each cycle, we found the majority (32) of transients going to the period 10 cycle. A skewing of the transient distribution to the periodic cycles (as opposed to the fixed points) is a characteristic we have noted when creating Boolean models of systems with the potential for chaos.

The second example is the cAMP receptor of *D. discoideum*, shown in Fig. 12 [Goldbeter, 1996; Aubry & Firtel, 1999]. This time the logic functions are:

$A_{(T+1)} = 1 + C_{(T)}$	(101)
$B_{(T+1)} = 1 + A_{(T)}$	(102)
$C_{(T+1)} = H_{(T)} + B_{(T)}H_{(T)}$	(103)
$\mathbf{D}_{(\mathrm{T}+1)} = \mathbf{C}_{(\mathrm{T})}$	(104)
$\mathbf{E}_{(\mathbf{T}+1)} = \mathbf{D}_{(\mathbf{T})}$	(105)
$F_{(T+1)} = 1 + E_{(T)}$	(106)
$G_{(T+1)} = D_{(T)} + E_{(T)} + D_{(T)}E_{(T)}$	(107)
$H_{(T+1)} = G_{(T)} + F_{(T)}G_{(T)}$	(108)

Noticing from the structural graph that node C (camp outside the cell) is the most centrally located in terms of its affect on other nodes, we look for a scalar equation for C and find that  $C_{(T+5)} = C_{(T)}(1 + C_{(T+2)})$ . This means that the value of C at T = 5 is determined by its value at T = 0 and T = 2, at T = 6 by its value at T = 1 and T = 3, etc. Therefore to get a complete enumeration of the dynamics of C requires examining all 32 different combinations of C at T = 0, 1, 2, 3, 4 as listed in the following table:

T = 0	0000000000000001111111111111111111111
1	00000001111111100000000111111111
2	0 0 0 0 1 1 1 1 0 0 0 0 1 1 1 1 0 0 0 0
3	001100110011001100110011001100110011
4	01
5	0000000000000000111100000111100000
6	0000000110011000000000011001100
7	0 0 0 0 1 0 1 0 0 0 0 0 1 0 1 0 0 0 0 0
8	0011001100110011000110000001100000011
9	01010101000100010101010100010001
10	00000000000000001111
period:	155555555555555555555555555555555555555

The final conclusion is that there is a single fixed point (1,0,0,0,0,1,0,0), and two distinct period five cycles:

and

Note that in both cycles the element C, cAMP outside the cell, varies. The excretion of cAMP in pulses is a hallmark of this system, so the variation of element C was an expected result and indicates that the Boolean model is a reasonable representation of the real biochemical system.

We determined numerically that the fixed point has few (21) transient states while each of the period five cycles has more than 100. Furthermore, like the previous example, neither of the periodic attractors have any frozen elements. Thus, this is a system that has negative feedback, periodic cycles with large basin of attraction, and no frozen elements in the periodic cycles. The marked similarity in the cycle and transient structures of the Boolean model of this system and the previous system suggests the possibility that this protein-protein interaction network might also exhibit either a limit cycle or chaos (or both) for different values of the system parameters.

## 6. Conclusions

We have shown in this paper that potentially large Boolean networks are amenable to exact analysis. The structure of a Boolean network is described in terms of its cycles and the transient states that lead to them. We have developed two different methods, iteration and scalar form, to determine cyclic structure. We have shown that the methods apply to a wide variety of different examples, many arising in biological and biochemical networks.

Reduction to scalar form has the advantage of simplicity when it is successful. Therefore, an obvious question is whether there are general conditions under which it will apply. It would also be very helpful if the cyclic structure could be determined from the form of the scalar equation, without the need to consider  $2^n$  initial conditions for an  $n^{th}$  order scalar equation. Obviously for large systems this will be a practical necessity. We have seen that this is true for simple scalar equations such as  $A_{(T+6)} = A_{(T)}$ ,  $A_{(T+6)} = 1 + A_{(T)}$ , and  $A_{(T+4)} = A_{(T+2)}A_{(T)}$ . We conjecture that this is true for more complicated scalar equations as well. It remains to be shown how general the form of scalar equations is for which this is true.

Our preliminary studies with these new analytical tools on a non-trivial biochemical network has given us a strong new hypothesis to test-- namely that the structure of a biochemical signal transduction network is such that there is the potential for chaos. Evidence for the productive use of chaos would provide a much needed understanding as to why biochemical signal transduction involves such a complex network of interacting components. The slime mold *D. discoideum* is studied because its signal transduction networks are very analogous (sharing many of the exact same components) to that of higher organisms. Thus the results and conjectures presented in this paper provide a new basis for the elucidation of the function of complexity in the living cell.

The development of size n analytical tools for understanding Boolean networks with n nodes (and subsequent computer implementation of these tools) is an important and challenging task which we continue to pursue in our efforts to model biological and biochemical systems. Alligood, K. T., Sauer, T. D., & Yorke, J. A. [1997] Chaos (Springer, NY).

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## Figure Legends

**Figure 1.** A simple Boolean network with three nodes and all OR logic. This network has two fixed points and no periodic cycles. A, the directed graph; B, the logic of the network; C, a map of all possible trajectories; D, the logic of the entire system condensed to a single table.

**Figure 2.** Another simple Boolean network. This network has two periodic cycles (of period 2 and 6) and no fixed points. A, the directed graph; B, the logic of the network; C, a map of all possible trajectories; D, the logic of the entire system condensed to a single table.

**Figure 3.** An extension of the linear network show in Fig. 2. Analysis (in the text) shows it has five period 12 cycles, one period four cycle, and no fixed points. A, the directed graph; B the logic of the network.

**Figure 4.** A nonlinear network with a period three and period six cycle. A, the directed graph; B the logic of the network.

**Figure 5.** Another nonlinear network. Although this network appears to be simpler than the one in Fig. 4, the analysis is actually more complex since the state of the network for the first three time points must be specified. A, the directed graph; B the logic of the network.

**Figure 6.** Another nonlinear network. This network has two fixed points and a single period 2 cycle. A, the directed graph; B the logic of the network.

**Figure 7.** An epigenetic model. This is a model of gene regulation by protein-DNA interaction. A, the directed graph; B the logic of the network.

**Figure 8.** A more complicated epigenetic model. A, the directed graph; B the logic of the network.

**Figure 9.** An idealized model of protein-protein interactions. A, the directed graph; B the logic of the network.

**Figure 10.** A Boolean network described by Glass and Pasternack [1978b]. The Boolean model has periodic cycles and, when modeled with differential equations, the network produces a limit cycle. A, the directed graph; B the logic of the network.

**Figure 11.** A model of coupled oscillations in the cell cycle. This system demonstrates chaos at certain parameter ranges. A, the directed graph; B the logic of the network.

**Figure 12.** A Boolean model of cAMP signaling in *Dictyostelium*. This well-studied biochemical system is known for producing cyclic aggregation signals.



Fig. 5

Fig. 6



Fig. 7



Fig. 9





Fig. 10





