Table 1. The effect of variation in the length of time between carcinogen and antigen treatment. The numbers in the parentheses represent the number of animals tested. Results are $\log_2$ of average hemolysin titer.

Time after immunization (days)	Interval between carcinogen and antigen (weeks)			
	Group	4	7–9	11
4	Treated	6.0(7)	6.6(8)	4.8(5)
4	Control	8.0(6)	9.8(10)	8.3(6)
		$P<.001$	$P<.001$	$P<.001$
10	Treated	6.3(3)	4.6(8)	4.8(8)
10	Control	7.0(3)	7.0(10)	6.8(8)
			$P<.001$	$P=.02$
19	Treated		3.5(8)	
19	Control		6.9(10)	
			$P<.001$	
30	Treated	4.3(9)		4.4(7)
30	Control	7.0(7)		7.7(8)
		$P<.001$		$P=.002$
60	Treated	4.1(8)		4.3(3)
60	Control	6.6(7)		6.5(4)
		$P<.01$		

mor incidence was only 53 percent for 76 mice injected with 30 μg and 19 percent for 47 mice receiving 10 μg. The incidence for mice receiving gelatin only was 4 percent. The first lymphomas began to appear approximately 10 weeks after treatment, and by 25 weeks virtually all mice that were going to develop a thymoma had done so. The three lymphomas that arose in 73 gelatin-injected control animals did not appear until 43 weeks, or more, after injection.

The whole-body weights of treated and control newborn animals were followed for 7 days. No differences were detected, a contrast to the results of Rappaport *et al.* (*4*) who observed a decrease in the whole body weight 4 days after the injection of 100 μg of DMBA.

To determine what tissues were being affected by the carcinogen treatment, the ability of the lymphatic system to respond to an antigenic stimulus was investigated. Mice, less than 24 hours old, received 60 μg of DMBA or, in the controls, 1 percent gelatin, and beginning at 4 weeks of age they were challenged with an antigen at various intervals. The preparation of the antigen and hemolysin assays have been reported (*5*). All titers are expressed as the logarithm to the base 2 (log 2) of the dilution (Table 1). Thus, a small dose of DMBA given within the first 24 hours after birth produced a significant and long-lasting reduction of the ability of the treated animals to respond to an antigenic stimulus when challenged as early as 4 weeks or as late as 11 weeks after carcinogen treatment.

A further attempt was made to obtain information on the site of action of DMBA with respect to its effect on the immunological response. It had been shown (*6*) that mice splenectomized as adults and challenged with a low dose of antigen administered intravenously do not give a detectable antibody response. Intraperitoneal injection of the antigen to splenectomized animals resulted in a response arising only from the extrasplenic lymphatic tissue. If splenectomy does not alter the response of the extrasplenic tissues, then it is possible to obtain information on the portion of the antibody response contributed by the extrasplenic tissue and, by difference, an estimate of the contribution of the spleen to the response. The results of this experiment indicate that 89 percent of the total response was contributed by the spleen as compared to 11 percent by the extrasplenic tissues. However, a comparison of the effect of 60 μg of DMBA on the responding tissues showed that both are affected proportionately to the same extent.

Malmgren *et al.* (*7*) showed that very large doses of chemical carcinogens injected into adult mice were able to depress the immunological response when the animals were challenged with an antigen administered during carcinogen treatment. The noncarcinogenic chemicals tested had no effect on the immunological response. Stjernswärd (*8*) has shown that 3-methylcholanthrene given in a large, single dose to adult animals 6 days before antigenic stimulation, not only depressed the hemolysin response but also resulted in a decrease in the number of antibody-forming spleen cells. Prehn and Main (*9*) have suggested that the impairment of the immunological response of the host is related to the ability of 3-methylcholanthrene to act as a carcinogen.

Thus, neonatal injection of the potent carcinogen DMBA depresses the capacity of the animals to produce hemolysins to sheep's erythrocytes, and it does not matter when the erythrocytes are given: the immunological capacity of the animal is chronically impaired throughout the duration of the latent period for the development of the thymomas. Possibly such chronic impairment plays an important role in carcinogenesis (*10*).

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Enzyme Amplifier Kinetics

Abstract. *Enzyme amplifier systems have been implicated in such diverse physiological phenomena as vision and blood coagulation. Such systems are considered from a general point of view and the concept of steady state gain of the enzyme amplifier is introduced. Expressions for the latter are obtained, and several of the main factors influencing gain are discussed. The effect of the duration of the activation on the characteristics of the response is discussed, and the influence of small changes in the rate constants on the steady state gain is considered.*

In recent years it has been suggested that certain important biological processes involve a sequence of enzyme-proenzyme reactions which exhibit a net chemical gain. Thus, MacFarlane (*1*) has proposed that blood clotting constitutes such an enzyme sequence. In this instance surface activation of comparatively few molecules of Hageman factor serves to activate further a series of some six additional factors which culminates in the conversion of millions of molecules of fibrinogen to fibrin. Wald (*2*) has also considered the possibility that vision may involve enzyme amplification since the

optical activation of a single molecule of rhodopsin can trigger a response involving a large number of molecules. Rhodopsin is postulated to be a proenzyme which is activated as a consequence of the absorption of a photon by 11-*cis*-retinaldehyde, resulting in the unmasking of the active site. Once formed, the enzyme can in turn activate a large number of molecules of the next proenzyme in the series, and so on down the chain with each step providing a gain in the number of participating enzyme molecules.

Both postive and negative feedback probably play a role in the regulation of enzyme amplifiers (*1*). In the case of blood clotting, positive feedback is provided by thrombin which seems to accelerate the reactions associated with the conversion of factors V and VIII. On the other hand, fibrin seems to accelerate the disappearance of the other clotting factors.

In order to facilitate the study of such enzyme amplifiers it is helpful to have available a mathematical model of the system. Consequently, in what follows such a model will be developed for an amplifier neglecting any feedback effects. In particular we consider a cascaded sequence of reactions for which, at the ith stage, a proenzyme of concentration y_i at initial concentration y_{i0} is converted by an enzyme of the previous stage to the active form of concentration y_{ia} at a rate determined by the constant k_i. The rate at which the activated form is destroyed is determined by the constant K_i (we assume none are equal) while the concentration of the inactivated form is designated by y'_i. This process is considered to be terminated at the Nth stage. The sequence may now be represented as follows.

$$\begin{array}{l} y_1 \rightarrow y_{1a} \rightarrow y'_1 \\ \quad\searrow \\ y_2 \rightarrow y_{2a} \rightarrow y'_2 \\ y_3 \\ \cdot \\ \cdot \\ \cdot \quad y_{(N-1)a} \\ \quad\searrow \\ y_N \rightarrow y_{Na} \rightarrow y'_N \end{array} \qquad (1)$$

If we assume that the first factor, y_1, is activated over some time interval $\Delta t = a$ after which the stimulus is terminated, then the differential equations describing the system are as follows.

$$\begin{aligned} \frac{dy_{1a}}{dt} &= k_1 y_1[U(t) - U(t-a)] - K_1 y_{1a} \\ \frac{dy_{2a}}{dt} &= k_2 y_2 y_{1a} - K_2 y_{2a} \\ &\cdot \\ &\cdot \\ &\cdot \\ \frac{dy_{Na}}{dt} &= k_N y_N y_{(N-1)a} - K_N y_{Na} \end{aligned} \qquad (2)$$

In the foregoing, $U(t)$ is the unit-step function, and hence $[U(t) - U(t-a)]$ is a unit pulse of duration a. It is possible to provide a general solution to (*2*) if the linear case is considered for which the concentration of proenzyme y_i remains constant at the initial value y_{i0}. This will be approximately so if the amount of activated proenzyme is small in comparison to that initially present or if the precursor is replaced at approximately the same rate as it is converted. The build-up in the active form is given by the solutions to Eq. 2:

$$y_{ia} = \frac{y_{10}k_1}{K_1}\left\{U(t) - U(t-a) + \exp(-k_i t)\,[\exp(K_i a) \times U(t-a) - 1]\right\} \qquad (3)$$

and for the pth stage

$$y_{pa} = \frac{y_{10}k_1}{K_1}\,\frac{y_{20}k_2}{K_2}\cdots\frac{y_{po}k_p}{K_p}\left\{U(t) - U(t-a) + \sum_{i=1}^{p} A_i \exp(-k_i t) \times [\exp(K_i a) \times U(t-a) - 1]\right\} \qquad (4)$$

where

$$A_i = \frac{1}{\prod_{j=1}^{p}\left(1 - \frac{K_i}{K_j}\right)} \quad (j \neq i)$$

It is informative to consider the above solution as the product of two factors one of which, $F(t)$, alone depends on time while the other factor, R, depends only on the initial concentrations and on the ratio of the rate constants. For $t \leq a$ the factor $F(t)$ increases from an initial value of zero to a steady state value of unity.

We note that the time required to reach the steady state, t_s, is determined by the smallest decay constant K_m and is given by the condition $t_s > K_m^{-1}$. When the system is at steady state we may define the gain G of the enzyme amplifier as

$$G = \frac{y_{aN}}{y_{a1}} = \prod_{i=2}^{N} \frac{y_{i0}k_1}{K_i} \qquad (5)$$

It is evident that if the time interval, a,

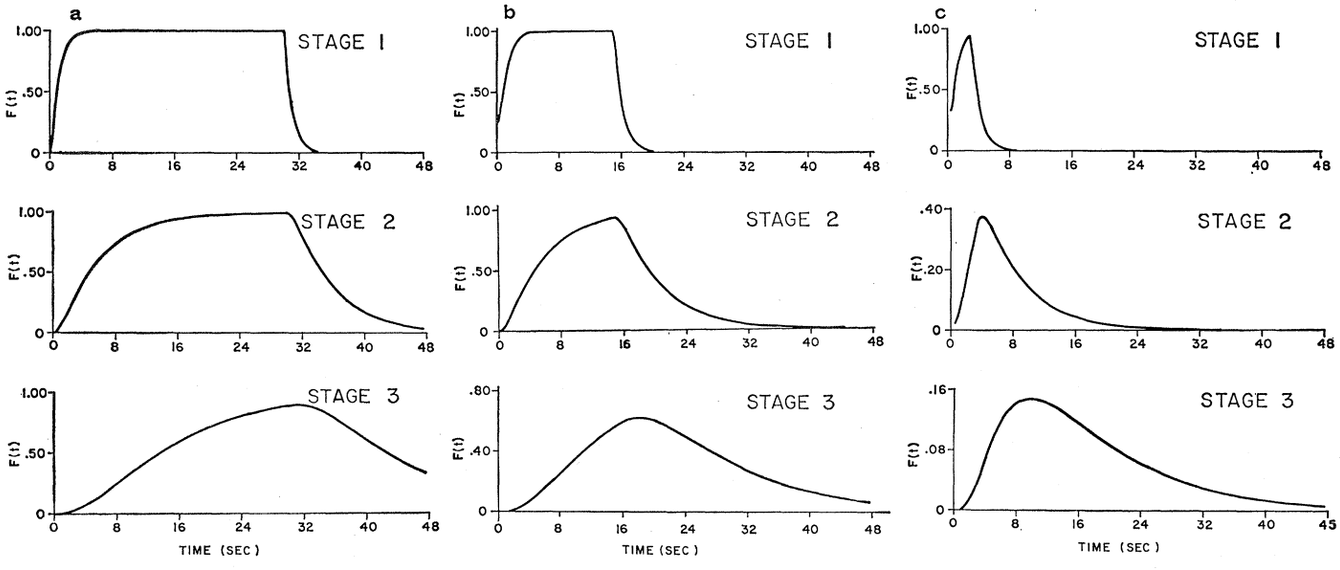

Fig. 1. The effect of varying time of activation of stage I of biochemical amplifier on the form and magnitude of the output response, stage 3. The time of activation decreases from 30 units in (*a*) to 15 units in (*b*), while (*c*) corresponds to 3 units. Here $F(t)$ is the time-dependent factor in Eq. 4.

over which y_{10} is activated is not sufficient for the realization of the steady states, then the gain of the enzyme amplifier will be less than that indicated by Eq. 5. The effect of progressively shorter time intervals, a, on $F(t)$ is shown (Fig. 1) in the instance of a three-stage process. Though the choice of constants is arbitrary the curves make clear the nature of this dependency.

A second point with reference to Eq. 5 is that the gain involves the products of several single-stage terms. This means that small shifts in the rate constants or the intial conditions can collectively produce significant overall effects. Thus a decrease of only 2 percent in k_i and y_{10} together with a corresponding increase in K_i results in an eight-stage process, such as blood coagulation, in an overall decrease in gain of nearly 40 percent. This sort of effect has bearing on negative and positive feedback which effects several stages simultaneously.

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Erythropoietin: Production by a Particulate Fraction of Rat Kidney

Abstract. *An erythropoietic factor was extracted with hypotonic phosphate buffer from the kidneys of hypoxic rats. Normal rat serum enhanced the activity of this factor, which is associated with the light mitochondrial fraction. The data suggest that the renal factor is not physiologically active unless it interacts with a serum carrier or activator, or that the factor may be an enzyme which produces erythropoietin from some serum substrate.*

Erythropoietin has been extracted from the kidneys of hypoxic and anemic rats. The extract obtained by homogenization in 0.02*M* phosphate buffer, *p*H 6.8 (*1*), contains a mixture of erythropoietin, some derived from residual plasma in the renal vasculature, and some of intracellular origin (renal erythropoietic factor). We now report details of the extraction and properties of the renal erythropoietic factor.

Thirty-two adult female Long-Evans rats were rendered hypoxic by continuous exposure to 0.5 atm of air for 17 hours. The animals were then immediately exsanguinated, and their kidneys (43.9 g) were removed, minced, and homogenized in 100 ml of cold isotonic saline (*p*H 7.0). The homogenate was centrifuged at 37,000*g* for 30 minutes, and the sediment was washed in 38 ml of isotonic saline. The isotonic supernatants were combined. The sediment was homogenized again in 150 ml of 0.02*M* phosphate buffer at *p*H 6.8, and the supernatant was collected by centrifugation. This two-step procedure resulted in separation of plasma-borne residual erythropoietin (isotonic supernatant) from the renal erythropoietic factor (hypotonic supernatant). Erythropoietic activity was assayed in mice rendered plethoric by discontinuous exposure to hypoxia (*2*, *3*). Values obtained for incorporation of radioactive iron into circulating red cells were converted to unit equivalents of standard A (*3*). All materials were administered intraperitoneally in a volume of 1 to 2 ml. The assay was sufficiently sensitive to detect as little as 0.02 unit of erythropoietin.

The hypotonic extract contained approximately 75 percent of the total erythropoietin recovered from the kidney (Table 1*A*). This indicates that the bulk of the erythropoietin in the kidneys of hypoxic rats may be contained within particles. Based on the method for estimating residual plasma volume (*1*), the activity in the isotonic saline extract was due mainly to residual plasma erythropoietin. Both extracts were incubated at 37°C for 30 minutes with an equal volume of normal rat serum. The erythropoietic activity of the hypotonic extract increased more than twofold after such incubation, whereas the isotonic saline extract did not (Table 1*A*). The normal rat serum contained no activity when assayed alone. This activation phenomenon is believed to be a characteristic property of the renal erythropoietic factor. Perfusates of kidneys of anoxic rats also enhance erythropoietic activity when incubated with normal serum (*4*). Association of the erythropoietic factor with the nuclei of kidney cells has been recently reported (*5*).

To clarify further the nature of the renal erythropoietic factor, a subcellular fractionation of the kidney was undertaken (*6*). Thirty-four kidneys (23.5 g) from adult female Long-Evans rats made hypoxic by exposure to 0.4 atm of air for 17 hours were used. The nuclear, heavy-mitochondrial, light-mitochondrial, and microsomal fractions were collected, and each fraction was resuspended in 50 ml of 0.02*M* phosphate buffer (*p*H 6.8) and centrifuged at 37,000*g* for 30 minutes. The supernatant of each fraction was then assayed for erythropoietic activity before and after incubation with an equal volume of normal rat serum. The extract of the light-mitochondrial fraction exhibited no detectable activity when assayed alone but showed the largest amount of erythropoietic activity after incubation with normal rat serum (Table 1*B*). This extract had a protein concentration of 8 mg/ml. Enhancement was noted only with the light-mitochondrial extract. It seems, therefore, that the renal erythropoietic factor is localized in particles intermediate in density between the heavy mitochondria and microsomes.

An increase in the time of incubation or the amount of light-mitochondrial extract results in a decrease in activity (Table 2). Such effects may be due to the hydrolytic enzymes—some of probable lysosomal origin—present in the fraction (*7*) since erythropoietin is known to be inactivated by proteolytic enzymes (*8*). Regression data show no significant deviation from parallelism between standard A-erythropoietin and the product of the interaction of the light-mitochondrial extract with normal rat serum. This implies that the product is physiologically indistinguishable from erythropoietin. Extracts of the light-mitochondrial fraction of 24.0-g kidneys from nonhypoxic rats were obtained, and these also engendered erythropoietic activity when incubated with normal rat serum (Table 1*C*).

Active renal extract, obtained from adult male Long-Evans rats made hypoxic by 17-hour exposure to 0.4 atm of air, was acidified to *p*H 5.0 and then quickly neutralized. This extract no longer evoked erythropoietic activity