that determine cell division. The meaning of these numbers has yet to be clarified. But the Monte Carlo method affords a means for estimating them from data on various aspects of individual cell life.

JOSEPH G. HOFFMAN
Biophysics Division, Rosewell Park Memorial Institute, Buffalo, New York
NICHOLAS METROPOLIS
VERNA GARDINER
Theoretical Division, Los Alamos Scientific Laboratory, Los Alamos, New Mexico

References and Notes

1. This work is supported by the U.S. Atomic Energy Commission.
2. S. Iversen and N. Arley, *Acta Pathol. Microbiol. Scand.* 27, 1 (1950).
3. H. B. Fell and A. F. Hughes, *Quart. J. Microscop. Sci.* 90, 355 (1949).
4. J. G. Hoffman, *The Size and Growth of Tissue Cells* (Thomas, Springfield, Ill., 1953).
5. D. G. Kendall, *J. Royal Statist. Soc.* B14, 41 (1952).
6. O. Rahn, *J. Gen. Physiol.* 15, 257 (1932).

7 January 1955

Nonbicarbonate Buffers in Cell Culture Media

The use of cell cultures in studying fundamental problems in many fields of experimental biology has increased considerably in the last few years. It is frequently desirable to employ cells that have been cultivated under conditions that are as standardized as possible. From the beginning of "tissue culture," cells of mammalian and avian origin have been cultivated *in vitro* in media composed of a balanced salt solution containing sodium bicarbonate as the principal buffer, supplemented with embryo extract and serum. Adequate control of the *p*H of such cultures introduces a number of technical problems, for it is necessary to maintain a specified carbon dioxide tension in the atmosphere by employing gassing procedures and closed culture flasks. In an attempt to circumvent some of these difficulties, studies were initiated to test the possibility of growing cells in media containing a fixed buffer instead of the conventional bicarbonate system. In addition to facilitating the control of *p*H and reducing the labor required for maintaining cultures, fixed-buffer media have a number of obvious applications in mass culture techniques and in metabolic and viral studies with cultured cells.

The following cell cultures were employed: fibroblasts derived from normal human foreskin, testicle, human embryonic kidney, and lung, as previously described (*1*); epithelial cells, strain HeLa (*2*); mouse fibroblasts, strain L (*3*). All cultures were grown directly on glass. The medium for foreskin, embryonic kidney, lung, and testicle cells consisted of mixture No. 703 (*4*) supplemented with 5-percent beef embryo extract and 10- to 20-percent horse serum. Human serum (20 percent) was employed instead of horse serum for HeLa cultures, and the L cells were grown on mixture No. 703 supplemented with 10-percent beef embryo extract.

The sodium bicarbonate of mixture No. 703 was replaced with tris(hydroxymethyl)aminomethane (5, tris) at concentrations of 2.0 to 3.6 g/lit, and the *p*H was adjusted to the desired value by the addition of hydrochloric acid. This mixture was supplemented with embryo extract and serum in the same concentrations as were employed for the stock cultures. Foreskin, embryonic kidney, lung, and testicle cultures grew as well as the controls (bicarbonate medium) with all levels of tris when the initial *p*H of the nutrient fluid is 7.8 to 8.1. All cultures were propagated serially with weekly subcultures. Foreskin and testicle cells have been maintained for the longest period and continue to do well after 3 mo. These cultures have been maintained in both stoppered flasks and in flasks closed only by means of cotton plugs or loose-fitting metal caps. A number of differences between cells grown in tris and those grown in bicarbonate have been observed. For example, it appears that the number of cells required per flask is a more critical factor in tris fluids than it is in bicarbonate fluids. The only change in the appearance of cells cultured in tris fluids noted to date is a small increase in the number of intracellular granules. The results of a limited number of experiments indicate that prolonged cultivation of cells in fluids containing tris does not alter their susceptibility to infection with polio virus or the yield of virus. In contrast with the growth rate of normal human fibroblasts, the growth rate of HeLa and L cells was somewhat inhibited with higher levels of tris. The HeLa cells were smaller and more granular than those in the control cultures and there was a tendency for the cells to grow in clumps rather than as a continuous monolayer. The L cells also exhibited changes in morphology when they were cultured in media containing tris. Despite alterations in morphology and decreased rate of proliferation, both strains have been maintained through 14 subcultures and continue to proliferate at a constant rate.

The results of short-term experiments indicate that glycylglycine is less toxic than tris for HeLa cultures. Under these conditions, the cells grew rapidly without any appreciable alteration in morphology. Cultures of foreskin cells, however, did not proliferate as well in glycylglycine as they did when tris was employed as the principal buffer.

Although the results obtained to date with tris are particularly promising with cultures of normal human fibroblasts, factors such as concentrations of tris, optimal *p*H, and the effect of other constituents of the medium require more detailed study before a final evaluation can be made. Several organic and inorganic compounds with a pK_a in the range of 6.5 to 8.5 that are compatible with other components of cell culture media are now under investigation in an attempt to replace tris in cultures where it is toxic or where the optimal *p*H for growth is below the effective limit of this buffer system.

H. E. SWIM
R. F. PARKER*
Department of Microbiology, School of Medicine, Western Reserve University, Cleveland, Ohio

References and Notes

1. H. E. Swim and R. F. Parker, *Proc. Soc. Exptl. Biol. Med.* 83, 577 (1953).
2. G. O. Gey, W. D. Coffman, M. T. Kubicek, *Cancer Research* 12, 264 (1952).
3. K. K. Sanford, W. R. Earle, G. D. Likely, *J. Natl. Cancer Inst.* 9, 229 (1948).
4. G. M. Healy, D. C. Fisher, R. C. Parker, *Can. J. Biochem. Physiol.* 32, 327 (1954).
5. G. Gomori, *Proc. Soc. Exptl. Biol. Med.* 62, 33 (1946).

* Aided by a grant from The National Foundation for Infantile Paralysis.

9 May 1955

Substrate Specificity of Peroxidase

A current view of enzyme specificity is that compounds of certain specific configurations can be bound by the enzyme and can lead to formation of the activated complex. The substrate specificity of catalases and peroxidases for hydrogen peroxide has been recognized for some time, and Theorell has recently suggested the term *hydroperoxidases* for this class of enzymes (*1*).

Such a definition of specificity includes reactions with homologs of the substrate, methyl or ethyl hydrogen peroxide, which have been shown to react in a second-order fashion with peroxidase and to form the same series of intermediates as does hydrogen peroxide (*2*). But the possibility of forming these reactive intermediates by a wide variety of inorganic oxidizing agents, recently pointed out by P. George (*3*), would seem to vitiate current ideas of formation of the enzyme-substrate compound and to favor the idea that the spectroscopically detectable peroxidase intermediate (complex I) is only the product of some rapid, unobserved oxidation reaction caused by a great variety of nonspecific oxidants.

This possibility lead George to the view that peroxide would react by a similar mechanism and would not therefore be a component of the structure of complex I, nor would it, per se, be required for

Table 1. Effect of bromoiridate concentration on the rate of disappearance of bromoiridate and the rate of appearance of complex I (0.01M borate buffer at pH 9.18).

Expt.	Bromoiridate (μM)	Peroxidase (μM)	Rate of disappearance of bromoiridate* (sec^{-1})	Rate of formation of complex I* (sec^{-1})
1	14	1.0	0.041	0.076
2	20	1.0	0.040	0.067
3	28	1.0	0.030	0.077

* First-order velocity constant.

the formation of this complex. But an alternative interpretation is that peroxide is the specific substrate for peroxidase and that either peroxide itself or a derivative thereof is formed as an intermediate in the reaction of the other oxidizing agents with peroxidase. On this basis one would expect that these other agents, when compared with peroxide itself, would produce complex I more slowly, and would show a less favorable stoichiometry (a large ratio of oxidant utilized to complex produced) because of participation in side reactions with the enzyme itself.

When hydrogen peroxide reacts with peroxidase, complex I is formed in a very rapid reaction that has been shown to be of the second order ($10^7 M^{-1} \times sec^{-1}$) over a 40-fold range of peroxide concentrations (*2*). The stoichiometry of the reaction is correct, 1 $H_2O_2 \rightleftharpoons$ 1 Fe atom of peroxidase converted to complex I (*4*).

The kinetics and stoichiometry of the reaction of peroxidase with two oxidizing agents, chlorite and hypochlorite, have been studied (*5*). More than 100 times the quantity of these reagents is necessary to give half-maximal formation of complex I than is required when hydrogen peroxide is used. Chlorite forms a peroxidase intermediate at one twenty-fifth the rate obtained with peroxide. Thus, in both cases, the formation of intermediate peroxide is possible.

One-electron transfer reagents such as chloroiridate provide a much more critical test of the reaction mechanisms, and George reported the formation of an intermediate resembling complex I with this reagent (*3*). We here (*6*) report kinetic and titration studies with bromoiridate, which has been found to be a more satisfactory reagent than chloroiridate (*7*). The formation of complex I was measured at 403 mμ and the disappearance of bromoiridate was measured at 510 mμ.

Kinetic data. Table 1 shows that the first-order velocity constants for the disappearance of the bromoiridate and for the appearance of complex I are independent of the oxidant concentration over the available experimental range; no second-order reaction of oxidant and peroxidase is obtained as in the case of hydrogen peroxide.

Titration data. Figure 1 shows that there is a nonlinear relationship between the amount of oxidant consumed and the amount of complex I produced. Larger amounts of bromoiridate give relatively less of the complex. The maximum slope of the titration curve corresponds to $>$ 4 $K_2IrBr_6 \rightleftharpoons$ 1 Fe atom of peroxidase converted to complex I. More than twice the theoretical amount of bromoiridate is required.

We found then that the formation of complex I from oxidizing agents other than hydrogen peroxide itself is either not a second-order reaction at all (bromoiridate) or is a second-order reaction over an order of magnitude slower than that caused by hydrogen peroxide (chlorite). The absence of a second-order rate law in the case of bromoiridate proves that intermediate reaction steps precede the formation of complex I. The slow second-order velocity constant in the case of chlorite allows time for intermediate reaction steps to occur. Mechanisms for peroxide production in similar reactions have been suggested by George (*8*), and other relevant possibilities are discussed by Evans and Uri (*9*). A practical aspect of the bromoiridate reaction is the finding (*7*) that this reagent attacks histidine as rapidly as it attacks peroxide and may act upon peroxidase by first combining with end groups of the protein. This would explain the unfavorable stoichiometry of the bromoiridate reaction ($>$ 4 $K_2IrBr_6 \rightleftharpoons$ 1 Fe atom peroxidase) (*10*). In view of these data and other aspects of the peroxidase and catalase reactions (*11*), it appears premature to revise current views of enzyme-substrate specificity, and it is suggested that the term enzyme-substrate complex be retained for the present.

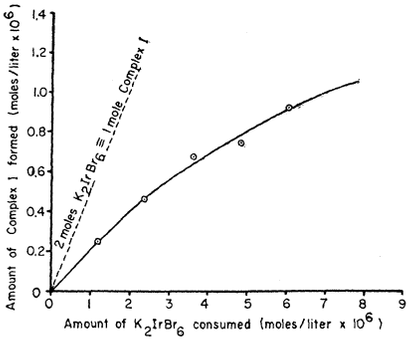

Fig. 1. Amount of complex I formed versus the amount of K_2IrBr_6 consumed. Initial (HRP): 1.19×10^{-6} moles $\times$ lit^{-1}; 0.01 moles $\times$ lit^{-1} borate (pH 9.2).

R. R. Fergusson
B. Chance
Eldridge Reeves Johnson Research Foundation for Medical Physics, University of Pennsylvania, Philadelphia

References and Notes

1. H. Theorell, in *The Enzymes*, B. Sumner and K. Myrback, Eds. (Academic Press, New York, 1951), vol. 2, part 1, p. 397.
2. B. Chance, *Arch. Biochem. and Biophys.* 22, 224 (1949).
3. P. George, *Science* 117, 220 (1953).
4. B. Chance, *Arch. Biochem. and Biophys.* 41, 416 (1952).
5. ———, *ibid.* 41, 425 (1952).
6. This work was supported in part by the National Science Foundation.
7. R. R. Fergusson, *J. Am. Chem. Soc.*, in press.
8. P. J. George, *Biol. Chem.* 201, 413 (1953).
9. M. G. Evans and N. Uri, *Symposia Soc. Exptl. Biol.* 5, 130 (1951).
10. Recently P. George has studied the oxidation of protein groups in hemoglobin by oxidizing agents [*J. Chem. Soc.* 1954, 587 (1954)].
11. B. Chance and R. R. Fergusson, in *The Mechanism of Enzyme Action*, W. D. McElroy and B. Glass, Eds. (Johns Hopkins Press, Baltimore, 1954), p. 389.

16 May 1955

Tetrapod Limb

I have just read with the greatest interest Grace Orton's note on the "Original adaptive significance of the tetrapod limb" (*1*). Romer (*2*) has suggested that the tetrapod limb arose as an adaptive modification that enabled the primitive amphibians to migrate from the receding pools of the Devonian countryside to areas where more water was retained. The tetrapod limb was an adaptation for terrestrial locomotion to permit the primitive amphibian to remain in the water. Orton has pointed out that such a suggestion seems to run counter to experience, for the behavior of modern amphibians is such that they will disperse only if the surrounding areas are sufficiently moist to attract them; if the adjacent areas are dry, modern amphibians tend to congregate in the dampest spots available. Such behavior is clearly the exact opposite to what might be expected were Romer's thesis correct, and Orton makes the alternative suggestion that the tetrapod limb was originally an adaptation for digging prior to estivation rather than for terrestrial locomotion.

Observations on the behavior of the South African Clawed Toad, *Xenopus laevis* (Daud.), together with some speculation, may help to throw some light on this problem. *Xenopus* is a typically