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.

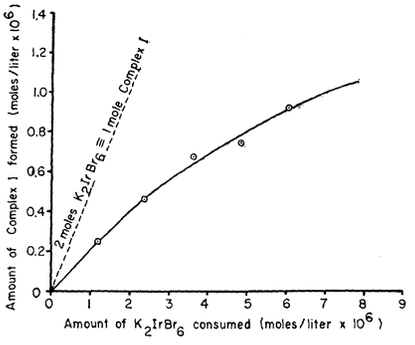

Fig. 1. Amount of complex I formed versus the amount of K_2IrBr_6 consumed. Initial (HRP): 1.19×10^{-6} moles $\times$ lit^{-1}; 0.01 moles $\times$ lit^{-1} borate (pH 9.2).

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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

aquatic animal, inadept at walking on land and very susceptible to desiccation. Of interest here are the various types of behavior shown by *Xenopus* in response to the drying up of its normal habitats. Three distinct types of pattern have been described, one of which, although relevant, is clearly unnatural. First, the toad may estivate by burrowing in mud or possibly by being simply entombed (*3*). Such protection is in full keeping with Orton's suggestion. Second, and equally in line with Orton's view, is an account (*4*) of *Xenopus* emerging from muds of a duckpond that had been cleared out during the dry winter season and spread over some neighboring lawns. With the first rains, numerous toads emerged from the muds and wandered aimlessly in all directions, despite the presence of the newly filled duckpond some 20 yd away. Most of the animals died from exposure to the sun. Clearly their enforced terrestrial emergence left the animals defenseless against desiccation. The third and most interesting event is the description in the same paper of a migration across land of several thousand clawed toads from a dam that was commencing to dry up towards a nearby river. Such a migration as this is by no means unique, for I have heard several reports of sections of a main road being littered with the corpses of *Xenopus* that had been killed by passing cars.

Such then are the facts. To turn to speculation, it seems, first, highly probable that such a mass migration occurs only in favorable climatic conditions. Unfortunately there are no records on this point, but for the present let it be allowed as a hypothesis. Second, it seems likely that this sudden pattern of migration was not released simply by the dam commencing to dry up, for that is a common event that normally does not result in migration. Some other factor leading to migration must therefore be sought and the most obvious hypothesis, in the absence of evidence, is that the stimulus for migration arises from population pressure. If this is conceded, then clearly, with slight modification, Romer's hypothesis can stand. Increasing aridity caused migration from drying swamps, but the immediate stimulus for such a migration was population pressure resulting from the reduction in size of the habitable area. The migratory pattern was released by the stimulus of population pressure, but only provided that conditions for migration were suitable. It is clear that this latter restriction on the release of the migratory pattern would be very rapidly selected. If these assumptions are allowed, then we need not picture a set of behavior patterns in the early amphibians totally different from those that we encounter today, for in *Xenopus*, which is normally aquatic and capable of estivation in soft mud, we also find migratory behavior of a type that could be conducive both to survival and to the selection of more efficient limbs.

Finally, Orton's hypothesis does not appear to offer us any explanation of why the tetrapod limb, evolved in her view in relation to burrowing, should suddenly be used for walking. If we accept her viewpoint we still have to discover a further selective agency that turned a fossorial appendage to the service of seemingly unnecessary terrestrial locomotion.

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23 May 1955

Effect of Hematoporphyrin on X-radiation Sensitivity in Paramecium

When a cell divides, it synthesizes the pigment, protoporphyrin, to supply the daughter cells with the necessary quantity of heme compounds. Precursors of the erythrocytes produce this pigment in abundance and attach iron and globin to manufacture hemoglobin. Leukocytes and all other cells also produce protoporphyrin and attach iron and protein to manufacture various respiratory enzymes such as the cytochromes, catalase, and peroxidases. The naturally occurring porphyrins are, therefore, found in relatively high concentrations in embryonic, hemopoetic, traumatized regenerating and neoplastic tissues, many of which are radiation sensitive. Moreover, it has been found that these radiation-sensitive tissues have a great affinity for injected hematoporphyrin (*1*). Although protoporphyrin must be present in every cell at some stage of its development, a greater amount is present in cells that are undergoing division (*2, 3*).

It was thought by us that some of the porphyrins might have an effect on x-radiation sensitivity. It, therefore, seemed desirable to test the effect of hematoporphyrin on radiation sensitivity of *Paramecium*, which, besides being a single, isolated cell, is also a complex organism. Because these organisms are so radioresistant and for other reasons (*4, 5*), they proved to be excellent material for this study (*6*). Paramecia in control media require exceedingly high dosages of x-radiation to produce lethal effects. For *P. caudatum* the approximate LD-50 of 340,000 r, 24 hr, is about 850 times as great as the estimated LD-50 of man and some common vertebrate laboratory animals (*5*).

It was known that hematoporphyrin sensitizes paramecia to visible and ultraviolet light (*3*). Our experiments demonstrate in a conclusive way that porphyrin sensitizes paramecia to short wavelength radiations, such as x-rays. Even in extremely high dilutions, hematoporphyrin-treated specimens proved to be approximately 18 times as radiosensitive as the controls.

For each irradiation experiment, 200 paramecia were placed in each of four nylon hypodermic syringes of 2-ml capacity and were x-rayed simultaneously. The paramecia were well-fed (*Aerobacter aerogenes* in standard desiccated lettuce infusion) vegetative specimens from rich clonal cultures. The plastic syringes in a Plexiglas holding and cooling chamber were placed between two crossfired x-ray tubes operated at 182 kv peak at 25 ma with an equivalent filtration of 0.2 mm of copper and a delivery rate of 6300 r/min. The syringes absorb very little radiation, permit the introduction of hematoporphyrin in various concentrations, and facilitate accurate sampling of specimens during and after irradiation without changing the depth of the medium.

The dilutions of hematoporphyrin were prepared so that when they were mixed with an equal amount of the culture fluid containing the paramecia, concentrations of 1:20,000 to 500,000 could be achieved. Concentrations of 1:10,000 hematoporphyrin are occasionally toxic to paramecia, unless they are kept in

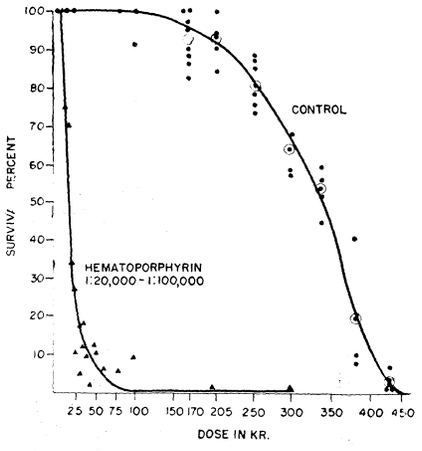

Fig. 1. X-ray survival curves for *P. caudatum* irradiated in nylon syringes. These curves are based on nine experiments. Each point in the curve represents observations on 10 to 25 counted specimens for at least 24 hr after irradiation. Control, ●; hematoporphyrin, ▲.