The antibody fixed to the antigenic components of the glomeruli might not be exposed to cells with such proteolytic powers. If the antibody were attached to the glomerular basement membrane, which has been shown to be the most antigenic of the kidney's components(11), it might even be extracellular and therefore not exposed to proteolytic attack by intracellular enzymes.

Conclusions. In the presence of specific antigen 62% of the homologous anti-protein antibodies are rapidly removed from the circulation within one hour after injection and 86 per cent within 6 hours. Once removed from the blood, the antibodies are promptly catabolized, between one third and one half within 6 hours and virtually all between 24 and 48 hours. In the rabbit the rates of catabolism of anti protein antibodies and heterologous serum protein antigens after their elimination from the circulation as specific complexes are comparable.

Since the submission of this manuscript for publication a paper dealing with this subject by S. P. Masouredis, L. R. Melcher and M. B. Shimkin entitled "The Effect of Antigen Administration on the Behavior of I^{131} Labelled Rabbit Anti-Ovalbumin in Mice" has appeared in the *Journal of Immunology,* 1953, v71, 268.

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Reproducibility of X-Ray Survival Curves for Yeast Cells.* (20673)

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While investigating the influence of "modifying factors" (anoxia, protective chemicals, temperature, and physical state) on radiosensitivity of yeasts(1), it was found that the changes in radiosensitivity that had to be measured were relatively small. This made it necessary to develop a technic that would yield accurate and reproducible X-ray survival curves and to investigate cultural and physical conditions that might influence the radiosensitivity of the cells and the shape of their survival curves. These investigations are described in this paper. The influence of other modifying factors will be reported elsewhere.

Methods. Unless otherwise specified, a haploid strain of the yeast *Saccharomyces cerevisiae* was used; in one experiment a genetically related diploid strain was used, both strains being supplied by Prof. R. E.

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Zirkle. Stock cultures on Difco potato dextrose agar (PD agar) slants were kept at —30°C. From these, monthly subcultures were prepared, incubated at room temperature (25°) for 4 days, and stored at 0°. Streak cultures were prepared from the subcultures daily and incubated at room temperature until used (14 days unless otherwise specified). Cells were harvested with a wire loop and suspended in M/15 KH_2PO_4 at a concentration of approximately 10^7 cells/ml. (Acid phosphate was used for all suspensions and dilutions.) After agitation the cells were washed by 3 centrifugations (2000 g) of 5, 1, and 5 minutes, respectively. The cells were resuspended in an Erlenmeyer flask and placed on a Boerner rotary shaker for 90 minutes, after which they were filtered through a 5-cm thickness of pyrex wool to remove large pieces of debris and clumps of cells that might be present. The cells were examined microscopically for clumping or budding, and a hemocytometer was used to count the number of cells/ml. After dilution to 10^6 cells/ml, approximately 200 ml of this suspension in a 500-ml flask were shaken for at least 3 hours. Samples were then taken from this stock suspension as needed. The X-ray machine was a General Electric Maximar, operated at 250 KV, 15 ma, with inherent filtration only. The exposure rate was 425 ± 5 roentgens/minute at the surface of the irradiated suspensions, as read on a Victoreen chamber placed in the same plane as the yeast suspensions. For irradiation, 1.2-ml samples from the stock suspension were placed in plastic-capped glass vials, 21 mm in diameter and 50 mm high. Not more than 3% of the X-ray beam was absorbed in traversing the yeast suspension. Uniform doses were attained by rotating the vials (80 rpm) in a horizontal plane 18 cm from the cone aperture of the X-ray machine in such a manner that the bottom halves of the vials were submerged in a bath kept at 20.0 ± 0.1°C. Control samples were kept in the same bath and were shielded with lead.

After the desired treatment (X-raying, etc.), each sample was diluted accurately, and 0.5 ml aliquots were spread with L-shaped glass rods on the surface of PD agar plates that had been poured 3 days earlier and allowed to dry. Dilutions were so made that 200 to 400 colonies would be formed per plate (except at very low survivals), a visible colony being taken as the criterion of a viable cell. Within one hour after plating (first buds appeared in 2 to 4 hours) plates were incubated at 30° ± 1° for 64 hours without further movement. Visible colonies were then counted and surviving fractions computed as the ratio of the number of cells/ml surviving X-irradiation to the number of cells/ml in controls. Usually 2 vials were used per experimental point and 4 plates per vial. The computed standard errors of the means were only slightly larger than sampling errors when survival was greater than 0.01, but increased somewhat for survivals less than this. For several survival curves the experimental points were fitted to an exponential curve by the method of least squares. However, since fitting by eye gave results to within 0.5% of those obtained by least squares, most curves were fitted in this way.

Results. Curve A of Fig. 1 is a standard X-ray survival curve for haploid yeast obtained by the technic described above. The standard errors of the experimental points are less than 2% (approximately the size of the points of the graph) and hence the shape is reliably determined. The experimental points can be fitted by an exponential curve‡ with considerable precision. In several experiments with haploid yeast, slight departures from the exponential relation, $s = e^{-nkIt}$, were noticed at low doses. Curve B of Fig. 1 shows the experimental points obtained in the most divergent experiment. The solid line through the points is theoretically obtained by considering that there are 2 classes of cells present: 1) normal haploid cells, and 2) "divided haploid" cells (cells that might be in nuclear division or might have completed division without visible budding; see *Discus-*

‡ Exponential survival curves are defined by the relation $s = e^{-nkIt}$; s is the surviving fraction of cells after a period of irradiation t minutes at a rate of I roentgens/minute, and n is the number of "sensitive sites" each of sensitivity k (see Zirkle and Tobias(6)). Thus nk is a direct measure of the radiosensitivity.

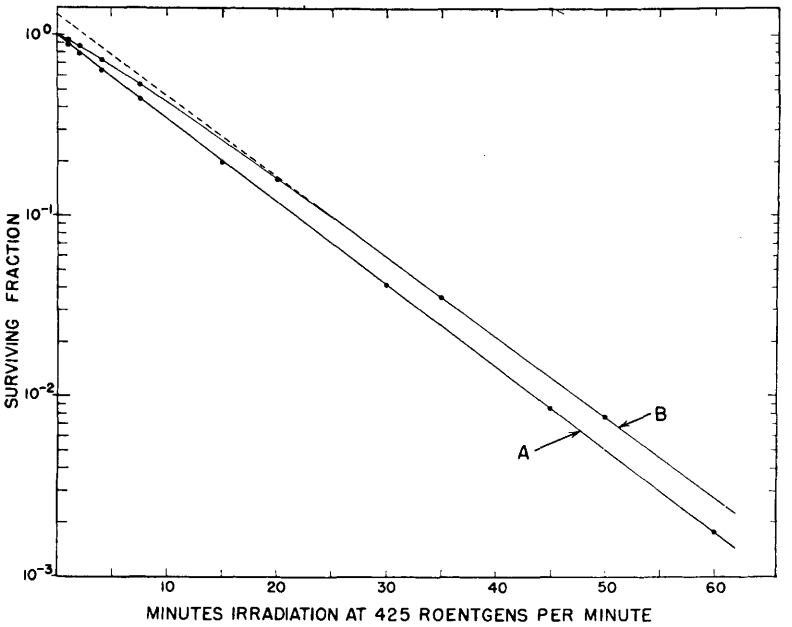

FIG. 1. X-ray survival curves of haploid yeast. Experimental points have errors of 2% or less (approximately the size of the points). Curve A: normal haploid survival, $s = e^{-0.106t}$ (t is irradiation time in min.). Curve B: contamination with "divided haploids," $s = 0.67e^{-0.103t} + 0.33\ (2e^{-0.103t} - e^{-0.206t})$.

sion). This analysis would also hold for the case of contamination with clumped or budded cells; microscopic examination, however, revealed neither. Furthermore, experiments showed that this departure from the exponential did not depend in a predictable fashion on the age of the culture from which cells were obtained. As but few haploid curves showed this small departure from the exponential, X-ray survival curves of the haploid yeast will be considered exponential.

That these results are reproducible over extended periods is shown by the data of Table I in which the radiosensitivities (nk) are given for typical experiments with 14-day cultures over a period of 18 months.

Several hours were often required for the completion of an experiment. However, aliquots of yeast suspension taken from a stock suspension (10^6 cells/ml) at various intervals up to 20 days showed no statistically significant change in either radiosensitivity or titer. Furthermore, cells irradiated at concentrations of 2.5 x 10^3 and 10^6 cells/ml displayed the same radiosensitivity although the titer of non-irradiated cells stored at the lower concentration dropped by a factor of 2 in 24 hours unless fortified with filtrate from a concentrated yeast suspension. Viability as used above is measured by colony formation from non-irradiated cells and does not necessarily mean that all cells in the controls are viable.

Microscopic examination, however, of

TABLE I. Reproducibility of X-ray Survival Curves for Haploid Yeast.

Date		nk × 10^4 (roentgens^{-1})	Deviation (%)
9–13–51		2.36 ± .05	2
3–21–52		2.46 ± .04	2
7– 8		2.52 ± .05	4
8–10		2.46 ± .03	2
2–23–53		2.26 ± .06	6
	Avg	2.41	

TABLE II. Dependence of Radiosensitivity of Haploid Yeast on Age of Culture.

Culture age (days)	Radiosensitivity, nk × 10^4 (roentgens^{-1})
2	3.58 ± .07
6	2.47 ± .05
14	2.41 ± .04
28	2.42 ± .04

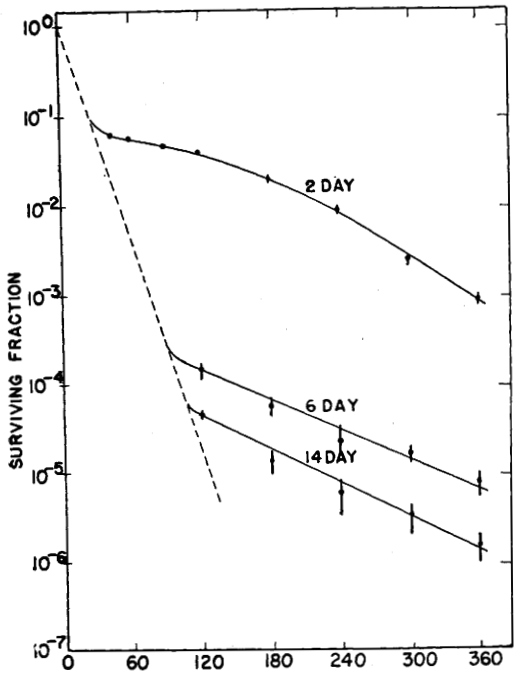

FIG. 2. "Tail" formation on x-ray survival curves as a function of age of culture. Dashed line represents initial exponential portion of each curve.

plated aliquots revealed that at least 98% of all cells prepared by means of the standard technic form visible colonies.

In Table II the results of a typical experiment are listed illustrating the dependence of the radiosensitivity, nk, on the age of the culture from which the stock suspension was prepared. Very young cultures (2-day) are considerably more sensitive than older ones. Often this is "explained" by the rule of Bergonie and Tribondeau(2) which states that the radiosensitivity of cells is directly related to their "activity"; generally young cells are considered more active. However, "starving" 2-day cells by agitation in acid phosphate for 48 hours before irradiation does not change their radiosensitivity although their activity should be reduced to a very low level by this treatment.

In Fig. 2 it can be seen that at high X-ray doses there is a definite break from the exponential part of the curve with the formation of a "tail" and that the older the culture (up to 14 days), the lower is the appearance of the tail on the curve. To test if the tail were caused by a genetically more resistant strain, X-ray survival curves were obtained for 32 cultures made from tail-survivors of a previous irradiation. In all cases the usual type curve was obtained.

To test if the tail position for young cultures could be varied, survival curves were obtained for a 2-day culture that had been "aged" by agitation in acid phosphate for 4, 24, and 48 hours. The point of origin of the tail remained remarkably constant although there was a small change in the shape and slope of the tail region, the curve for the more "aged" cells showing increased concavity downwards. Careful attempts to determine if there were any photoreactivation of X-ray inactivated yeast gave negative results. Latarjet(3) has reported an increased survival of X-rayed *Saccharomyces ellipsoideus* when stored in the cold before incubation. However, storage at 5° of X-rayed haploid yeast used in these experiments for periods up to 7 days before incubation gave decreased rather than enhanced survivals.

In Fig. 3 the experimental points of an X-ray survival curve are shown for the related diploid strain of yeast. The solid

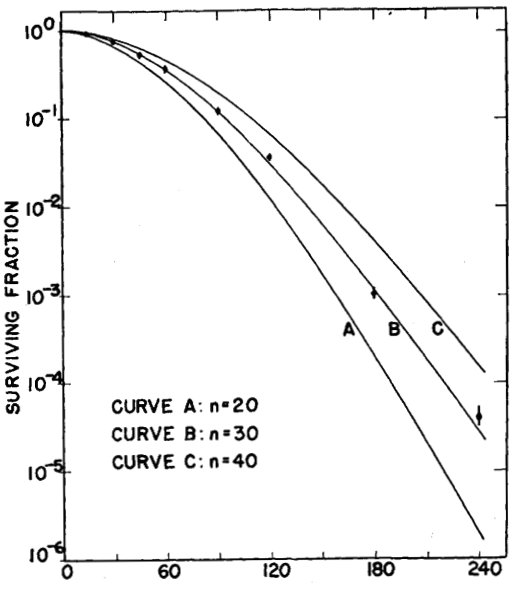

FIG. 3. X-ray survival points of diploid yeast. Curves A, B, and C are computed from the relation $s = (2e^{-kIt} - e^{-2kIt})^n$, with n given the various values shown.

curves were obtained from theoretical considerations that will be discussed later.

Discussion. It is desirable to place the test organisms in such a state that they form a homogeneous population. There are at least 2 ways to approach this. 1) Use a growing but statistically homogeneous population from a device such as a "chemostat"(4). 2) Use a resting population such as would be obtained if cells were kept on agar slants for long periods. Here it would be hoped that the cells would have approached a common, resting state. Cultures grown by the first method display considerable variation in the size of individual cells and there is a great deal of budding and clumping; these factors might easily influence the radiosensitivity. However, cells harvested from the surface of PD agar after 6 days or more of growth show no clumping, less than 0.1% budding, and are approximately uniform in size (4 to 5 μ in diameter). For these reasons cells were grown on agar slants.

Cells prepared as described in *Methods* appear to be in a steady state condition as determined by the consistencies in the radiosensitivity and in the titer. Hence time *per se* can be eliminated as a disturbing factor for suspensions of 10^6 cells/ml.

The individual experimental points of most published survival curves for various radiations have errors of 10% or more, and the curves themselves are not very reproducible. However, the above methods allow points of X-ray survival curves to be determined quite accurately; thus it is possible to ascertain the shape of the curves with considerable exactness. The normal haploid X-ray survival curve (Curve A of Fig. 1) for a 14-day or older culture is exponential down to survivals of 10^{-4} or less. This is interpreted as indicating that inactivation of haploid yeast by X-rays is due to a single event.

Occasional departures from the exponential type curve (Curve B of Fig. 1) do not necessarily conflict with the above interpretation. A possible explanation of Curve B is the following. In any population of yeast some of the cells might be in or might have completed nuclear division without visible bud formation. These divided haploids, having 2 sets of chromosomes, would be more resistant to irradiation. Consider the simplest possible case: a fraction f_2 of the population has undergone complete nuclear division without bud formation. Then the fraction $f_1 = 1 - f_2$ of the population is normal.§ The survival of such a mixed population to X-rays would be given by $s = f_1 e^{-nkIt} + f_2(2e^{-nkIt} - e^{-2nkIt})$. The slope nk can be obtained from the low survival region of the curve. An excellent fit is obtained (the solid line of Curve B, Fig. 1) by assuming that f_2, the fraction of divided haploids, is 0.33 ± 0.05 in this experiment.

No adequate explanation can be suggested for the dependence of the radiosensitivity on the culture age (Table II). Although growing, young cells might be more "active" than resting, old cells, a pretreatment similar to the one used here was shown by Aldous, Fisher, and Stern(5) to reduce carbon dioxide production and oxygen consumption to an endogenous level. Certainly the 48-hour agitation in acid phosphate of 2-day cultures should have changed the radiosensitivity (the initial slope) to that characteristic of older cultures if cellular activity alone determined the radiosensitivity. It is entirely possible that the "cross-sections" for the pertinent inactivation processes depend on the previous metabolic history of the cell. For example, it may be that older cells accumulate higher concentrations of cellular products that could serve as protecting agents against diffusing poisons produced by irradiation.

The tail effect (Fig. 2) has previously been reported by Zirkle and Tobias(6) and by Latarjet and Ephrussi(7), although it was observed at much higher survivals (around 0.05). Possible explanations for this difference are: 1) yeast were probably not placed in a state of endogenous metabolism; 2) irradiations were carried out on blocks of agar instead of in aqueous suspensions; 3) a different criterion of viability was used by Zirkle and Tobias (microscopic colonies of 3 or more cells instead of visible colony formation); 4) a different strain of yeast may have been used by Latarjet and Ephrussi. The

§ This is, of course, an oversimplification; there would be many intermediate cases.

fact that the "tail" regions are approximately exponential suggests that 2 populations are involved; the first (the larger) has the radiosensitivity characteristic of the initial part of the curve, the second, that of the tail region. This second fraction depends on the age of the culture. That these resistant cells are not genetically stable was revealed by the fact that cultures grown from them have the radiosensitivity characteristic of normal cells. From the experiments in which young cells were "aged" in acid phosphate, it appears that this resistant fraction remains constant under resting conditions.‖ It is interesting to note that while young cultures have a larger fraction of radioresistant cells, the rest of the cells are more sensitive than in older cultures (Table II).

Zirkle and Tobias(6) have estimated the number of "sensitive sites" in the haploid yeast necessary for continued cell division. It is worth while to repeat their calculations using these more accurate survival curves. The haploid curves provide data for determination of nk. The theoretical diploid X-ray survival curve is given by the expression $s = (2e^{-kIt} - e^{-2kIt})^n$, where the symbols are as before. In Fig. 3 the above function is plotted for assumed values for n of 20, 30, and 40, nk being obtained from the haploid curves. A very good fit to the experimental points is obtained for $n = 30$. Hence, under the given experimental conditions, inactivation of both members of any one of 30 ± 5 pairs of "sensitive sites" in the diploid cells results in inability to form visible colonies. Presumably this is also the number of "sensitive sites" in the haploid cell.

The fact that good exponential X-ray survival curves were obtained for the haploid strain down to survivals of 10^{-4} or less (for 14-day or older cultures) indicates that the method used here has certain advantages over those of Zirkle and Tobias and of Latarjet and Ephrussi. Also studies on effects due to temperature, protection, anoxia, etc., can be more easily carried out in aqueous suspension. The goodness of fit of the experimental points to an exponential curve is good evidence that the rate limiting process of inactivation is first order, *i.e.*, single event as postulated by Zirkle and Tobias in their diffusion model(6). Although the culture age has considerable influence on the radiosensitivity, exponential inactivation was observed for all ages. Other modifying agents have been used for this yeast system; Birge and Tobias(9) found yeast more resistant under anaerobic conditions; Wood(1) has investigated the effects of various temperatures and of phase state on the radiosensitivity. In all cases, although considerable changes in radiosensitivity were observed, the survival curves were exponential. These results are most easily explained by use of the diffusion model of Zirkle and Tobias and are good evidence that a sizeable fraction of the inactivation is due to indirect action.

Summary. Accurate and reproducible X-ray survival curves were obtained for both haploid and diploid yeast under carefully controlled conditions. The haploid curve is exponential down to survivals of 10^{-4} or lower for old cultures; the diploid curve is sigmoid and a good fit can be obtained by use of the proper multitarget model. The slope of the haploid survival curve (the radiosensitivity) depends on the culture age, young cultures being more sensitive. The haploid curves exhibit "tails" at low survival levels; the position of the tail is also dependent on the culture age, young cultures showing the tail at higher survival levels. The goodness of fit of the experimental points of the haploid yeast survival curves to an exponential curve is evidence that the rate-limiting inactivation step is first order as postulated in the diffusion model of Zirkle and Tobias.

The author would like to thank Professor R. E. Zirkle for research facilities and encouragement during this work.

‖ Beam, *et al.*,(8) have reported a correlation between the fraction of cells composing the "tail" and the number of cells with buds. They attribute the increased radioresistance of the buds to a duplication of nuclear material.

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Potentiation of Pteroylglutamic Acid by Ascorbic Acid in Anemia of Scurvy. (20674)

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The citrovorum factor (CF) is an active metabolite of pteroylglutamic acid (PGA) (1-3). CF replaces PGA in the nutrition of certain microorganisms(4,5) and animals (6,7), reverses aminopterin toxicity(2,6,8), and is effective in the treatment of nutritional macrocytic anemias in man(9-13). The conversion of PGA to CF by rat liver slices(14) or by resting cells of *S. faecalis* A(15) is enhanced by ascorbic acid. Excretion of CF in the urine following PGA injection is increased by ascorbic acid administration in normal man (16,17), whereas in adult human subjects with scurvy the ability to convert PGA to CF is impaired(18). CF is more effective than PGA in treating the megaloblastic anemia occurring in monkeys fed scorbutic diets low in PGA content(19,20). These observations suggest that ascorbic acid deficiency may precipitate certain nutritional anemias by limiting the conversion of PGA to CF. From studies on 2 patients with scurvy and anemia data are here presented which demonstrate a potentiation by ascorbic acid of the effect of PGA upon erythropoiesis.

Methods. Hemoglobin concentration, hematocrit, and red blood cell count were determined every 3 to 4 days by standard technics. Reticulocyte counts were done daily by the dry method(21), and at least 1,000 cells were counted for each determination. Buffy coat ascorbic acid levels were measured by the method of Butler and Cushman(22). The 24-hour urine outputs were collected and stored without preservative in a refrigerator; upon completion of each daily collection, aliquots were taken and stored at −10°C for assay. The PGA and CF activities of these urines were determined by microbiologic assays which utilize the growth response of *L. casei*(23) and of *L. citrovorum*(1,24), respectively. The levels of vit. B_{12} in the serum were assayed by a modification of the method of Ross which utilizes the growth response of *Euglena gracilis*(25). Serum iron concentrations were determined by the method of Kitzes *et al.*(26) employing o-phenanthroline.

Clinical observations. Case 1. A 42-year-old white male, gave an 8 months' history of inadequate food intake, particularly of ascorbic acid containing foods. On admission, he was weak, pale, and poorly nourished. He had a prominent perifollicular petechial rash over the arms, legs, and trunk, and several large ecchymoses on the posterior aspects of both legs. The tourniquet test was positive, but other coagulation studies, including bleeding time, clotting time, and clot retraction, were normal. Ascorbic acid was not present in the buffy coat of the venous blood. Hematologic studies revealed a macrocytic anemia with a hemoglobin of 6.6 g %, a hematocrit of 21.6 vol %, and a red cell count of 2060000/mm^3. The bone marrow aspirate contained a hyper-

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