KINETIC STUDIES ON THE MECHANISM OF PHOTOREACTIVATION OF BACTERIOPHAGE T2 INACTIVATED BY ULTRAVIOLET LIGHT

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George Hamilton Bowen, Jr.

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Abstract

Bacteriophage particles are called active if they are capable of generating a plaque when plated on agar by a standard technique. Exposure of the particles to ultraviolet radiation of wave length 253.7 mμ inactivates them in this sense. Following adsorption of the inactive particles to sensitive host bacteria, exposure of the suspension to light of the violet and near ultraviolet region causes a fraction of the particles to regain their activity, a phenomenon called photoreactivation.

The kinetics of photoreactivation of bacteriophage T2 have been investigated for the purpose of studying the mechanism by which photoreactivation takes place. The presence of a dark reaction in addition to the light reaction has been demonstrated. The dark reaction precedes the other and has the function of supplying the light-absorbing material which enters into the light reaction. Both the light and the dark reactions follow first-order kinetics.

The amount of photoreactivation produced by a given light treatment is determined by the interaction of the light and dark reactions. This interaction can be described satisfactorily in terms of a simple model for the reaction mechanism.

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I. Introduction

1. Inactivation by ultraviolet radiation.

Given a suspension containing bacteriophage particles, one commonly assays for the number present by plating a small volume of a suitable dilution together with a large number of sensitive host bacteria on nutrient agar. The presence of each active particle is recognized after incubation by the formation of a plaque, or clear area, surrounded by otherwise heavy and confluent bacterial growth.

If the phage particles, suspended in a medium transparent to ultraviolet light, are exposed for a short time to a source of ultraviolet energy and again assayed, it is found that only a fraction of the initial population remains active, i.e. able to give rise to plaques. The remainder of the particles, now inactive in this sense, can easily be demonstrated by other procedures to be still present and to retain various of their original properties, but the distinction between "active" and "inactive", based on plaque counts, is experimentally clear cut and leads to no ambiguities. It is this property of bacteriophage which concerns us here.

Our measure of activity is then the ability of the individual phage particle to invade a host bacterium, to produce likenesses of itself, and to lyse the host, releasing its offspring; these, by repetition of the cycle, form a plaque. The point is not known at which ultraviolet irradiation interferes in the chain of events leading from parent to progeny. In fact this chain of events, which one calls reproduction, is itself virtually unknown except for a few features relating mainly to its extreme ends.

Various phenomena are known which relate clearly to the first stages of invasion of the bacterial host by normal phage particles. Wherever the information is available, ultraviolet-inactivated particles are known to remain capable of each of these functions (except after very prolonged irradiation). These include adsorption to the host cell (1), destruction of chromatinic bodies of the host (2), killing of the host as a colony former (1), stimulation of the host to exclusion of related phage types which infect at a later time (1), lysis from without (3), and lysis inhibition of cells previously infected with r^+ types (3).

Following these early steps comes the "eclipse period" of the phage cycle, of which little is known, and this is ended in the latter part of the latent period by the appearance intracellularly of the first mature particles (4). Despite their apparently normal start, inactive phage particles never reach this destination. One is led to believe

that the ultraviolet action is associated with the most intimate and most obscure details of reproduction. It is for this reason that ultraviolet inactivation is a phenomenon of surpassing interest.

2. Nature of the immediate action of the ultraviolet radiation.

Knowledge of the immediate action of the ultraviolet radiation is very limited. For the killing of Staphylococcus aureus phage (5), of vaccine virus (6), of influenza virus (7), and of Escherichia coli (7) and other bacteria the action spectrum is known and seems definitely to implicate nucleic acid as the light-absorbing substance (for discussion and additional references, see Giese (8)). There is reason to believe that the same substance is responsible in the case of phage T2 inactivation. Moreover, the action is apparently a direct one on the nucleic acid itself, immediately causing some chemical alteration which is the end result of the irradiation. There is no reason to suppose that the nucleic acid passes on the absorbed energy to any other substance of the phage, that it is converted into a toxic form which then acts further on another substance, or in fact that there are any auxiliary reactions whatever. The effectiveness of the irradiation is only slightly affected by temperature (9). No chemical means of affecting the inactivation is known. So far as is known

there is reciprocity of light intensity and exposure time; their product, the total energy, alone determines the effect. This information is negative in character, however, and the picture must be further filled in by considering what is known of the function of nucleic acid in the phage, and of the photochemistry of nucleic acid.

Phage particles of type T2 are composed almost entirely of protein and desoxyribonucleic acid (DNA). Taylor found 45% of the dry weight of T2 produced in synthetic medium as DNA (10). Its importance in phage reproduction has been emphasized by recent work of Hershey and Chase with type T2 (11). They found that shortly after adsorption of an active particle to the bacterium the DNA is injected into the cell, and the residual protein "coat" of the phage can be removed without interfering with phage reproduction. Not more than 1% of the phage protein which can be identified by radioactive sulfur labeling remains with the bacterium. Conversely, phage "ghosts", prepared by subjecting particles to osmotic shock, have lost most, or perhaps all of their DNA and with it the ability to reproduce, but retain the ability to adsorb to the host and to produce various of the usual initial changes in it. These results leave no doubt of the close association of the DNA with the most essential phases of reproduction of the phage particles, and it is precisely these phases which seem to be affected in the case of ultraviolet inactivation.

The essential role played by the DNA is further attested to by the work of Hershey et al. (12) with phage particles incorporating radioactive phosphorus, which labels only the nucleic acid. Working with type T4, which is very closely related to T2, they found that about one radioactive disintegration in 11.6 led to inactivation of a particle, and that the inactivation proceeded exponentially. This rate could not be accounted for by the beta radiation produced. The inactivation must therefore have been a direct result of the nuclear reaction changing phosphorus to sulfur, or, since all the phosphorus is present in the DNA, the inactivation must have resulted from the chemical alteration of the DNA. Hershey et al. suggest that the particle can be thought of as containing a "vital structure" made up of about 8% of the total DNA, within which a single damage at any point is lethal.

Knowledge of the photochemistry of nucleic acid is regrettably small. Its absorption in the region near the wave length 260 mμ is responsible for the effect we observe. This absorption is due to the purine and pyrimidine residues it contains (13). Ultraviolet irradiation of solutions of DNA produces changes in the viscosity, solubility, light absorption, and behavior during dialysis and electrophoresis. These changes apparently result from photolysis of internucleotide links, but the process is poorly understood. Errera (14) reports finding a very low quantum yield (of the

order of 10^{-6}) for this photolysis. Errera also found photodecomposition of the pyrimidines during ultraviolet irradiation of the DNA, and this is probably the effect of greatest interest. After irradiation the DNA was hydrolysed, the pyrimidines isolated, and their amount measured spectrophotometrically; the actual structural changes involved in the destruction are not known. Errera states that the quantum yield is about 0.0025.

Zelle (15) gives the quantum yield for inactivation of phage T2 as 0.00015. However, if only the "vital structure" found by Hershey et al. is of importance, the quantum yield for this structure would be about 12 x 0.00015 = 0.0018, which is suggestively close to the value 0.0025 found by Errera for pyrimidine destruction. This apparent correspondence may be entirely accidental, but it is tempting to conjecture that inactivation of phage T2 by ultraviolet light results primarily from the photochemical destruction of pyrimidines of its DNA.

3. Photoreactivation.

Direct studies of the action of the ultraviolet light promise at best to tell us the nature of the immediate chemical alteration produced. The mechanism by which this alteration manifests itself as a damage interfering with normal biological functions remains unknown. Hope for additional understanding, particularly relating to this

second aspect of the problem has come with the discovery in the last few years of several reactivation phenomena, in which the expression of the ultraviolet damage is partially reversed. Of these the most striking and most general is photoreactivation (PhR), the reversal of the ultraviolet damage by light of longer wave length. It was first described by Kelner, who worked with conidia of Streptomyces griseus (16). Shortly thereafter, Dulbecco discovered the effect in bacteriophage (17), and it has since been found in a wide variety of organisms (see, for example, 18, 19, 20, 21).

Phage is particularly favorable material for experiments with PhR, and Dulbecco quickly gave a rather complete account of its behavior (22). The majority of the work was done with type T2. PhR of phage occurs only after adsorption to sensitive host bacteria, but it does become possible very quickly thereafter. The pigment responsible for absorbing the light energy is not known, but the action spectrum is approximately known and shows a single maximum in the vicinity of 365 m . PhR can take place with resting bacteria in the absence of external metabolites; oxygen is not necessary; cyanide does not inhibit. No chemical means of affecting the reaction is known.

If a suspension of phage particles is exposed to ultraviolet radiation, the fraction of active particles decreases from 1.0 to a lower value determined by the dose. If,

following adsorption of the particles to bacteria, the suspension is exposed to reactivating light, the active fraction increases to a new, much larger value; the fraction always remains less than 1.0, however. The transition of each individual from inactive to active status is clearly recognizable: either it generates a more-or-less normal plaque, or it gives none at all.

Dulbecco found that with phage T2 the increase in plaque count with time, t, of exposure to light is closely described by the function $(1 - e^{-at})$. The constant a may be called the rate of reactivation. This rate of reactivation does not increase linearly with light intensity, but tends to a maximum, or limiting value, and it is strongly affected by temperature. Neither of these facts would be true if the light reaction step were the only rate-controlling factor. Dulbecco was led, therefore, to infer the existence of a thermochemical, or "dark" reaction step in the PhR mechanism.

In order to demonstrate such a dark reaction more explicitly Dulbecco suggested experiments with intermittent light, and these were undertaken by the author. The first experiments gave the expected results and led into the detailed investigations which are to be described. These studies have given a clear picture of the light and dark reaction phases of the mechanism and of their interaction. They are an attempt to exploit this approach to learn as

much as possible of the nature of the reaction mechanism. Moreover we believe they are an essential preliminary to effective work from other approaches.

II. Materials and Methods

All experiments to be described have been done with bacteriophage T2r adsorbed to cells of Escherichia coli, strain B. The general experimental procedure has been almost identical in all cases. Individual experiments differ only as to the nature and conditions of the particular light treatment being tested. Information on the light treatment itself will be detailed carefully in each case with the experimental results.

1. Phage stock.

The bacteriophage stock is a crude lysate of T2r on B in the synthetic glucose-ammonium medium M9 (NH_4Cl, 1.0 g; KH_2PO_4, 3.0 g; Na_2HPO_4, 6.0 g; NaCl, 0.5 g; $MgSO_4$, 0.1 g; H_2O, 1000 ml; 4 g per liter glucose added after separate sterilization). The lysate was partially purified by one step of low-speed centrifugation to remove bacterial debris. In use it is always highly diluted, so there was no need to purify it further. The titer is quite stable, with a value of 4×10^{10} particles per ml.

2. Ultraviolet irradiation.

Phage particles from the stock are diluted appropriately in buffered saline, and a small quantity (2 ml) of the dilution placed in a watch glass, in which it is exposed to

radiation from a 15-watt General Electric "germicidal" lamp, 80 cm distant. This is a low-pressure, mercury-discharge lamp emitting a large part of its energy at 253.7 mμ. A convenient measure of the lamp's effectiveness is that 10^{-3} survival of T2r is reached with 47 seconds of irradiation. The timing of the irradiation is simply accomplished by uncovering and covering the solution with a lid opaque to ultraviolet light (the top of a petri dish), which is sufficiently accurate to give good results.

The buffer solution used here and elsewhere in the procedure is about 1/14 molar in phosphate, pH 7.0, with added sodium chloride and magnesium sulfate (Na_2HPO_4, 7.0 g; KH_2PO_4, 3.0 g; NaCl, 4.0 g; $MgSO_4$, 0.2 g; H_2O, 1000 ml). All the preparations are stable in it. It is a good adsorption medium for T2.

The irradiated phage particles are stable in their characteristics and can be kept in a cold room, in the fashion of normal phage stocks, for periods at least as long as a month. The surviving fraction and the behavior of the particles in PhR do not seem to change appreciably. Nevertheless, we have preferred to use freshly irradiated phage preparations, because this seems less open to possible criticism.

The ultraviolet dose has been chosen to give a surviving fraction in the range $1\text{-}5\times10^{-3}$ in all the experiments to be described, which represents a compromise between opposing

considerations. On one hand, it is desirable to use large ultraviolet doses (low survivals), because the ratio of survival in light to survival in dark becomes large and all effects are more easily observed. On the other hand, at low survivals it becomes increasingly difficult to avoid excessive amounts of multiplicity reactivation (23), which takes place in multiply-infected bacteria, and this covers up the most interesting features of PhR. This can be avoided only by using very low average multiplicity of infection; the practical limit is fixed by the minimum useful number of plaques per plate, and the maximum suitable bacterial concentration.

Typical experimental conditions representing a suitable compromise are an ultraviolet dose giving a survival of 3×10^{-3}, a bacterial concentration of 1×10^{8} per ml, and an average multiplicity of infection of 1×10^{-3}. This means that there are 1×10^{5} singly infected cells per ml, of which 3×10^{2} are active plaque formers in the dark, and 5×10^{1} multiply infected cells per ml (calculated from a Poisson distribution), most of which are active due to multiplicity reactivation. Plating 0.1 ml will then give about 35 plaques, of which only about 5 are a result of multiplicity reactivation. Three replicate plates will give a total of about 105 plaques, which is about the minimum useful figure inasmuch as statistical fluctuations in the counts become relatively quite large when the counts are small. (The

standard deviation with an average count of n is $n^{1/2}$.) Under these conditions the additional active plaque formers which appear during PhR come entirely from the class of singly infected bacteria. The increase in plaques can be followed directly, without the necessity of further dilution before plating, since several hundred plaques can be counted accurately per plate.

The plaque count before PhR can be increased without other change by increasing both bacterial concentration and phage concentration in the same proportion so that the average multiplicity of infection remains the same. Many of the experiments have been done with about 2×10^8 cells per ml, but the suspension is then quite turbid and becomes much more so at higher concentrations. Where accurate knowledge of the light intensity in the suspension is essential the bacterial concentration must be reduced, and this can only be done by increasing the multiplicity of infection and hence the proportion of multiply infected cells. At 2×10^7 cells per ml, average multiplicity of infection 5×10^{-3}, and ultraviolet survival 3×10^{-3}, there are still 30 plaques per plate from singly infected cells, but now there are 25 multiply infected cells per plate, and this is about the practical limit which still permits clear-cut and unambiguous experiments.

There have been small changes in these parameters from experiment to experiment according to the consideration

which seemed most important to the purpose at hand. In general the values have always been within the limits that have been indicated. A few early experiments were done at survivals as low as $1x10^{-4}$, and these, although less satisfactory, seemed to indicate that the mechanism of PhR found in the narrow range otherwise adhered to is not peculiar to one ultraviolet dose alone.

3. Bacteria.

The bacteria are grown in Difco tryptone broth, at 37° C, with aeration, to a concentration of about $2x10^8$ cells per ml, which is near the end of the log phase of growth. The cells are washed by sedimenting them in a centrifuge, discarding the supernatant liquid, resuspending them in an equal volume of buffer solution, recentrifuging, and resuspending them a second time in fresh buffer.

On some occasions, to reduce possible detrimental changes during their preparation, the bacteria were quickly chilled, washed as rapidly as possible in a cold room with cold solutions, and used immediately. However, in two experiments a portion of these carefully washed bacteria were starved by vigorous aeration at 37° C for periods of one and two hours, then infected, used for PhR, and the results compared with those from the fresh bacteria used immediately. Following starvation there was some loss of bacteria, both as colony formers and as phage yielders, and some decrease

in the maximum rate of PhR, but no effect was found in any of the essential characteristics of PhR. We conclude that the condition of the bacteria is not critical. Accordingly, in most cases the bacteria have been prepared as quickly as is convenient (about 30 minutes) and used promptly, but no other special precautions have been taken.

4. Adsorption.

The ultraviolet-treated phage particles are added to the bacterial suspension in the buffered saline solution, which is then kept at 37° C for 12-15 minutes to allow completion of adsorption to the host cells. The development of the ability to photoreactivate during this period was followed in two experiments which will be described in section III; after this time there is no further change. At normal temperatures the infected bacteria are satisfactorily stable and show only very small changes even when deliberately mistreated (as above); in the cold they can be kept without change certainly for many hours and probably for days. This adsorption mixture forms the starting point for the experiment proper.

5. Exposure to reactivating light.

Identical 0.5 ml samples from the suspension of infected bacteria are placed in each of a number of small irradiation tubes. Thereafter, all these tubes are treated

as nearly identically as possible, except in one respect: the light treatment each receives. Each tube, with its sample, is subjected separately to some specified light exposure, and thus gives an independent determination of the amount of PhR produced by that treatment.

6. Assay for active infective centers.

After PhR the count of active infective centers of a sample remains constant, so the time of assay is not critical. The most convenient procedure, and the one giving the best results, is first to complete the schedule of exposures for all samples, storing each sample in the cold and darkness after PhR, and then to plate all samples in short order at the close of the experiment. The time between PhR and plating may be hours if need be.

Several plates are made from each sample by standard agar-layer technique (25) using tryptone agar. As indicated previously, the concentration of infective centers is always so adjusted that a suitable number of plaques is produced by plating 0.1 ml directly from the irradiation tube without any intervening dilution step. Plaques are counted after incubation of the plates overnight at 37° C; the count reported for each sample is the total from all the replicate plates. Total counts for different samples can be compared directly as an indication of the PhR produced by each given treatment.

7. Equipment used for photoreactivation.

All work is necessarily done in a room illuminated only by non-reactivating yellow or red light. The common yellow-orange "insect-repellent" bulbs are suitable. The equipment used to obtain PhR is shown schematically in figure 1, and will be described briefly.

The light source is a 1000-watt projection lamp cooled by forced-air ventilation. It is operated from the 60-cycle line voltage with a series rheostat, which can be used to control the intensity.

In preliminary experiments with interrupted light the lamp was operated from a DC source and the light beam chopped by a rotating toothed wheel. These experiments showed that within the available accuracy interrupted light is equivalent to continuous light of the same average intensity for chopping frequencies from one cycle per second to 2400 cycles per second. It is now known that all rate-determining steps of PhR are slow compared to these frequencies, so that rapid variations in the light intensity are completely averaged out. The use of the more readily available AC power is therefore justified in spite of the fact that it introduces a 120-cycle ripple in the lamp output.

The light passes through a filter cell containing a solution of copper sulfate ($CuSO_4$, 23 g per liter in water; 1 3/4 inches thick), which removes the large amounts of red and infrared energy emitted by the lamp. Its transmission

is over 90% from 340 to 510 mμ, less than 1% above 680 mμ. In order to dispose of the large amount of energy absorbed by the filter solution, cool water is run through coils of copper tubing included in the filter cell.

The apparatus is enclosed in a light-tight housing up to the point of the shutter, which is used to control the time of exposure. The shutter is simply a vane moved by an electrical solenoid; it operates quickly and effectively and is easily controlled. In two experiments, where very short exposures were required, a camera shutter was used instead. The actual exposure time at each shutter setting was measured, and the correct times are recorded with the data.

A fraction of the total light is reflected downward to a phototube by a small piece of glass. The tube is a type 935 vacuum phototube; this has type S-5 spectral response, peak sensitivity at about 340 mμ, chosen as the response most similar to the PhR action spectrum. The light intensity is monitored continuously by measuring the phototube current.

It is clear that changes in emission from the lamp ordinarily involve changes in the spectral distribution as well as in the total amount of the energy. The changes in intensity as indicated by the phototube and as seen by the PhR pigment are not necessarily the same because their response as function of wave length is not identical. For small intensity changes the correspondence of phototube

current with PhR effectiveness is probably close, but for large changes the two almost certainly diverge widely, and the phototube current would then give a gross misrepresentation of the effective intensity. The lamp is therefore operated so as to give constant phototube current at all times, and intensity reduction at the sample is accomplished by the use of a rapidly rotating disc in which open sectors have been cut. It is placed just outside the shutter in the path of the light. Various such discs are available with sectors of different sizes giving different average intensities, and the ratios of the intensities are accurately known. The frequency of the flashes so produced is not synchronous with 60 cycles. The standard intensity will always be referred to as a relative value of 1.0.

The beam of light is reflected upward by a prism and enters the sample through the bottom of the irradiation tube. At this point the light beam forms a uniform spot slightly larger than the bottom of the tube. The maximum intensity that can be reached (relative value 1.25), as measured by a General Electric model DW-60 radiation meter (a thermocouple device), is about 2.5 calories per cm^2 per minute.

At the close of the present work a set of interference filters became available. The transmission of two of these lies well within the wave length range useful for PhR and is shown in figure 2. These were not used in any experiments, but the light energy passing through them was measured,

providing a useful, though very approximate indication of the actual amount of energy available for PhR. At the position of the sample, with maximum lamp intensity (1.25), the radiation meter registered about 0.025 and 0.02 calories per cm^2 per minute for energy received through the filters with peak transmission at 379 mμ and 401 mμ respectively.

The irradiation tubes are ordinary flat-bottomed, screw-cap vials of 5/8-inch diameter. They are selected from stock to have uniform diameter and flat bottoms. With that precaution, any variability introduced by differences among the tubes is not greater than other experimental errors. The variance of the plaque counts from a series of identical samples usually corresponds well with that expected in small numerical samples on the basis of chance alone.

The tube is held accurately in position by a spring clamp, and can be rapidly removed or inserted. The water bath surrounding the sample is thermostatically regulated to within ± 0.05° C of the stated temperature. This is accomplished by a mercury regulating device controlling a heating element in the bath. The water in the bath is stirred vigorously at all times by an electric motor. At low temperatures fogging of the outer surface of the bath vessel is prevented by directing a stream of compressed air at the point immediately below the sample, through which point the light passes.

III. Experimental Results

We shall first consider briefly the general character of PhR kinetics as found in experiments with long exposures of reactivating light leading to maximum PhR. This will then serve as a background for the detailed studies which form the main body of our work, and which concern exclusively the initial phases of PhR before the maximum is approached.

1. Maximum photoreactivation; approximate over-all kinetics.

Figures 3a and 3b show data from an early experiment in which PhR was measured as a function of the time of exposure to reactivating light. It is a repetition of the type of experiment described by Dulbecco and confirms his results. It illustrates well the general character and magnitude of the PhR effect.

The equipment used in this case was not the same as that described in the previous section and used in all subsequent work, but this is of no consequence since the experiment is introduced only as a background for the later work. The light source was a medium-pressure mercury-discharge lamp, type A-H5. The volume of solution illuminated was 5 ml; its temperature was 37° C. Exposure was begun at time zero, and samples were taken at time t, diluted, and plated. Data are plotted in figure 3a as the surviving fraction

relative to the active phage present before ultraviolet irradiation; survival in the dark, before PhR, was 5×10^{-4}.

The population of infected bacteria can be thought of as containing three classes of individuals: those active as plaque formers even in the dark (P_o), those capable of becoming active through PhR (ΔP_{max}), and those inactive in either case. The total active plaque formers at any given time will be called P. The data are described very closely (but, as we shall see, not perfectly) by the equations

$$\Delta P = (P - P_o) = \Delta P_{max} (1 - e^{-at}) \qquad (1a)$$

$$\ln\left(1 - \frac{\Delta P}{\Delta P_{max}}\right) = -at \qquad (1b)$$

where a is independent of t. In figure 3b the data are shown plotted as $\ln(1 - \Delta P/\Delta P_{max})$ vs. t. A straight line fitted to the points by the method of least squares intersects the vertical axis at 1.05 and passes within about 5-7% of all the experimental points; this error corresponds to about the statistical fluctuations expected in the plaque counts.

The simple exponential function which appears to describe the over-all kinetics of PhR led to the supposition that PhR is "one-hit", or one-event in nature (22). However one-hit repair of the multiple-hit ultraviolet radiation damage presents a paradox that has not been satisfactorily explained. This will be discussed in greater detail

in section V. We suggest that the repair does occur damage by damage and that the approximate one-hit reactivation of the particle as a whole is an artifact resulting from a variation among the damages of the rate at which they are repaired during exposure to light. The manner in which this can occur is shown in the appendix. The data of figures 3a and 3b do not coincide perfectly with equations (1a) and (1b), but show a small inflection near the origin. Evidence of such a deviation has been found repeatedly and will be pointed out in the experiments as they are presented; there is no doubt that it exists. Its magnitude is probably large enough to make possible an explanation on the basis of the assumption we have suggested.

In the first seconds or minutes of exposure to light have also been found other much larger effects which provide important clues to the nature of the mechanism by which light energy is received and utilized for PhR. These effects, which are the subjects of our experiments, are not obscured by the small deviations from linearity mentioned above.

2. Transient initial period: the light reaction.

At the beginning of a period of continuous illumination the rate of reactivation is directly proportional to the light intensity and only slightly dependent on the temperature. At high light intensities, however, the rate quickly decreases in an exponential fashion from the initial value

to a new, lower value, which thereafter remains constant, and PhR continues to a maximum in the manner previously described. The time constant for the decay of the initial rate is approximately inversely proportional to the light intensity and is almost independent of the temperature. In contrast, the second, or steady-state rate during continuous illumination is not proportional to the intensity and is strongly affected by temperature. The steady-state rate will be discussed in detail in part 3 of this section.

We believe that a clear picture of the primary photochemical reaction, isolated as a single reaction step, is to be found in this very brief initial period: that the initial rate of reactivation is directly proportional to the rate of the light reaction step under all conditions; that the decrease in the rate of reactivation, which is observed at high light intensities, is due to the depletion of light-absorbing material by the light reaction; that the first-order kinetics found for this decay in rate, and the time constant of the decay are the kinetics and the actual time constant of the light reaction step itself.

Data illustrating the characteristics of the initial period and its relation to the steady-state period following it are shown in figures 4, 5, and 6. Figures 4a and 4b present the results of an experiment at 37.0° C, plotted on two different time scales. Each sample received four light flashes, each of length t and intensity I, spaced at inter-

vals of three minutes. Under these conditions the effect of each flash is approximately equal, so that successive flashes serve to multiply the amount of PhR without changing its character. The otherwise small initial effects are thus increased relative to the number of survivors in the dark. (Effects concerned with intermittent light are considered in detail in part 4 of this section.) There were three independent samples exposed at each flash length; the total plaque count on two replicate plates from each sample is plotted as a function of the flash length.

In this and in the following experiment two different light intensities were used. Reduction of the intensity was accomplished by reducing the lamp voltage. It was not possible to include in the optical train the rotating disc ordinarily used for intensity reduction due to the presence of the camera shutter which was required for short exposures, and which had to be placed in the usual position of the disc. In order to have a valid measure of the intensity at the wave lengths useful for PhR, the ratio of the two intensities used was measured through the two interference filters previously described. This ratio was 7.2. On the scale of relative values that has been used the higher intensity was 1.25, and the lower was therefore about 0.175.

The smooth curves drawn in the figures are of the form

$$\Delta P = (P - P_o) = Rt + \Delta P_1(1 - e^{-At}), \qquad (2)$$

with the constants R, ΔP_1, and A chosen to fit the data as well as possible. It will be seen that the fit is very good. R is the steady-state rate; its characteristics and their interpretation will be discussed in part 3 of this section. ΔP_1 is the increase in plaque count due only to the initial phase of PhR and is obtained by extrapolating the steady-state line to zero time; it results from the utilization of that amount of pigment initially present which is in excess of the steady-state concentration. A is the rate constant for the completion of the initial phase; it will be shown in section IV that it should equal the sum of the light reaction rate and the competing dark reaction rate, but at high light intensities the former is dominant and A is approximately proportional to the intensity. The experimental results at 37.0° C are: I = 1.25, (1/A) = 0.95 second; I = 0.175, (1/A) = 5.7 seconds. A decrease in I by a factor of 7.2 thus decreases A by a factor of 6.0.

An identical experiment was performed at 0.0° C, except that the flashes were separated by 45-minute dark intervals to allow time for complete recovery between flashes. The results are shown in figures 5a and 5b. The steady-state rate, R, is very much lower than at 37° C and is not measurably different for the two intensities used, indicating that the dark reaction is extremely slow at this low temperature and now determines the steady-state rate completely. The initial rate of reactivation and the constant A are exactly

proportional to the light intensity. The results at 0.0° C are: I = 1.25, (1/A) = 0.6 second; I = 0.175, (1/A) = 4.3 seconds. The decrease in I by a factor of 7.2 decreases A by a factor of 7.2. The reason for the apparent small increase in the light reaction rate at the lower temperature as compared to that at 37.0° C is not known.

In figures 6a and 6b data are presented from an experiment designed to show the relation of the brief initial period to the over-all PhR curve. In this case each sample received only one light flash of length t and intensity I = 0.6. The temperature was 36.5° C. The adsorption mixture was divided into two portions, one being used directly for samples exposed for short times, and the other being diluted by a factor of 10 and used for samples exposed longer times leading to large amounts of PhR.

3. Steady-state rates during continuous illumination: interaction of light and dark reactions.

Following the brief initial period there is established a steady-state condition which, if illumination is continued, prevails to the completion of maximum reactivation. At low light intensities the steady-state rate of reactivation is directly proportional to the intensity indicating that the light reaction remains the limiting factor; but as the intensity is increased the rate of reactivation fails to increase proportionally and finally tends toward a fixed

maximum which is dependent only on the temperature. In this extreme condition the rate limiting factor is a dark reaction, whose function we believe to be the replenishment of the light-absorbing material depleted during the initial period. At intermediate light intensities the rate is determined by the interaction of both light and dark reactions which together determine the steady-state concentration of the pigment.

We shall define the rate of reactivation, R, as the slope of the line obtained in plotting plaque counts, P, *vs.* time of exposure, t, during the steady-state period. As such it is to be distinguished from the rate constant *a* defined by the relation,

$$\Delta P = \Delta P_{max} (1 - e^{-at}). \qquad (1a)$$

We shall confine all measurements to the first part of the curve, which is essentially linear, and in which

$$R = \frac{dP}{dt} = a \cdot \Delta P_{max} \qquad (3)$$

Thus R is directly proportional to *a* although it is given in the arbitrary units of plaques per unit time. Within any one experiment values of R found under different conditions can be compared as a measure or the dependence of *a* on these conditions.

It is found experimentally that the dependence of R on light intensity, I, is described satisfactorily by the

function

$$R = R_{max} \left(\frac{I}{I+B}\right) , \qquad (4a)$$

where R_{max} and B are appropriate constants. The function obviously has the desired qualitative features: with I much less than B it is directly proportional to I, and with I much greater than B it tends to the value R_{max} independent of I. R_{max} is thus the maximum, or limiting rate, and B is the intensity at which half maximum rate is reached. The interpretation to be developed in section IV is that R_{max} is proportional to the rate of the dark reaction producing the pigment, and B is the intensity at which the light reaction and the reverse dark reaction remove the pigment at equal rates.

Quantitatively the function is best checked against the data by plotting (1/R) vs. (1/I), which should give a straight line, since equation (4a) can be put in the form

$$(1/R) = (1/R_{max}) + (1/R_{max})(B/I) . \qquad (4b)$$

Both constants can be read directly from such a plot, for the vertical intercept is $(1/R_{max})$, and $(1/R) = 2(1/R_{max})$ when I = B.

Experiments have been performed to test this relation over the temperature range 0-45° C, and it appears to hold in all cases. The experiments also show that B changes rapidly with the temperature. Data are presented in figures 7-21, and a summary of the values found for B is given in

table 1.

In each case the direct experimental data are plotted first as P vs. t; then R is calculated for each intensity and a plot of (1/R) vs. (1/I) made. A straight line is fitted to the latter points by the method of least squares and the values of R_{max} and B read off. In all the experiments the general procedure was exactly that which has already been described. Each sample was placed at the indicated temperature, allowed to remain long enough for equilibration of temperature and the completion of the known dark reaction steps (see part 4 of this section), and given a single light flash of length t, intensity I. All samples remained the same length of time at the given temperature.

In order to avoid the risk of losing light intensity by absorption within the sample, the bacterial concentration was reduced to about 2×10^7 cells per ml in all cases. The optical density at 365 mμ of the bacterial suspension at this concentration is about 0.05, as measured in a Beckman spectrophotometer. Transmission through the 3-4 mm deep layer in the irradiation tube should then be about 96%. Moreover the loss of light measured by the spectrophotometer is almost entirely due to scattering, whereas most of the scattered light is not lost for PhR. There has been no indication that the effective intensity is significantly decreased even at cell concentrations of 2×10^8 per ml, so the lowering of the concentration to 2×10^7 per ml is

certainly an adequate precaution.

It is to be noted that whenever the intensity becomes much less than B, the value required for half maximum rate, a small lag appears at the beginning of PhR. This is the same effect found in the experiment of figure 3 and commented on in that case. At high intensities it is hidden by the large initial increase in plaques.

These experiments do not give a sufficiently accurate comparison of R_{max} at different temperatures. For this purpose a second series of experiments was made, in each of which the rate was measured at the standard light intensity, I = 1.0, both at the experimental temperature and at a reference temperature of 36.6° C. The ratio of these two rates can be determined accurately and is reproducible. The standard intensity produces a rate, R(1.0), within a few per cent of R_{max} at all temperatures, so the measured ratio can be corrected to the true ratio of the values of R_{max} with accuracy using the values of B obtained in the previous series of experiments. The necessary correction is:

$$R_{max} = (1+B) \cdot R(1.0) . \qquad (5)$$

Experimental data are shown and the calculation briefly indicated in figures 22-30. The values obtained for R_{max} are summarized in table 2.

If the number of particles capable of PhR is independent of temperature, then according to equation (3) both a_{max} and R_{max} should change with temperature in the

same way. Knowledge of a_{max} at any one temperature, which can be obtained only by following the complete kinetic curve to maximum PhR, would then make possible calculation of a_{max} at any desired temperature using the much more easily measured value of R_{max} at that temperature. The experiment of figure 6, which was at 36.5° C, indicated that (1/a) = 9 minutes at I = 0.5, and therefore that $(1/a_{max})$ = 8 1/2 minutes, approximately. This value can be used for approximate calculations of a_{max} at other temperatures, but it must be remembered that such calculations rest on the assumption that ΔP_{max} is constant.

4. Effects with intermittent light: the dark reaction.

In the darkness preceding illumination certain equilibrium conditions exist. Upon exposure to light these conditions are altered and a new steady-state condition is established which is sustained as long as illumination is continued. This is evidenced by the rapid decrease in reactivation rate from the high initial value to a low steady-state value found during exposure to high-intensity light.

If, now, the light is turned off and a considerable dark period is allowed, the initial state is restored, and a second exposure to light repeats exactly the pattern of the first. The cycle can be repeated indefinitely, although, of course, maximum PhR is rapidly approached. Figure 31

shows data from an experiment at 36.5° C with repeated flashes of light of various lengths separated by a constant dark interval of two minutes. The first flash is slightly less effective than those following, an effect apparently of the same origin as the small initial lag observed in figure 3 and at low intensities in figures 7-21.

By varying the length of the dark intervals between successive flashes it is possible to follow the progress of the dark reaction in time. Figures 32-38 show the results of a series of experiments of this type at temperatures from 0° to 45° C. Every sample received exactly the same light exposure, which was three 5-second flashes at intensity I = 1.0. The samples differed only in the length of the dark intervals, t_D, which separated the flashes.

At each temperature the data fit an equation of the form

$$\Delta P(t_D) = P(t_D) - P(0) = \Delta P(\infty)(1 - e^{-Ct_D}), \quad (6)$$

where P(0) is the plaque count with zero dark intervals (single continuous flash), $\Delta P(\infty)$ is the increase in plaque count found with very long dark intervals, and C is a specific rate constant. In each experiment a value of C is determined, and these values are summarized in table 3.

Since a single 5-second flash at standard intensity is just sufficient to almost exhaust the supply of pigment present, but allows little time for the dark reaction to

proceed (see figures 4 and 5), it is essentially a method of assay for the amount of pigment present at the time of the flash. With zero dark intervals the second and third flashes produce little PhR. The observed increase in plaque count with increase in dark interval length is then a direct measure of the restoration of light-absorbing material in the cell, and shows that the dark reaction producing the material follows first-order kinetics.

The amount of pigment present after a long dark interval is determined by a temperature-dependent equilibrium. If the temperature is changed we should expect this amount to change somewhat, and if the temperature change is abrupt the time required for the establishment of the equilibrium condition at the new temperature should be the same as for the recovery of the system at that temperature in the dark interval following an intense light flash, since the same dark reactions govern both. The effects following temperature changes are small and much more difficult to observe, but we believe the expectation has been verified at least in the most extreme case.

Figure 39 shows data from an experiment investigating effects following a temperature change from 40° C to 0° C. At time t = 0 the sample was moved from 40° C to 0° C, and at time t it received a single 10-second flash of light at intensity I = 1.0. At time t = 60 minutes it was returned to 40° C, equilibrated 15 minutes, and the cycle repeated in

order to multiply the amount of PhR. There were three such cycles in all. Thus all samples received precisely the same temperature treatment and the same light treatment, and differed only in the timing relations between the two. The time required for the temperature of the sample actually to reach 0° C was certainly less than one minute, but no samples were exposed at t less than one minute in order to avoid any questions of interpretation. The maximum change which could be produced was about 30%, but this was reproducible. The amount of reactivation became constant after about 30 minutes. This corresponds well with the rate of recovery at 0° C during the dark interval following a light flash, which is shown in figure 38.

If ample time for equilibration is allowed before the light exposure, and the effects of a temperature change after the light are investigated, no change is found. Repeated experiments with temperature changes after the light flash, whether an increase or decrease of temperature, whether one or several cycles, and no matter how carefully or rapidly accomplished, have always given completely negative results. We are forced to conclude that any steps required for PhR which take place in buffered saline after the primary photochemical step are either completed within a few seconds or are completely temperature independent.

5. Relation of photoreactivation to the adsorption process.

We have confirmed Dulbecco's result that no PhR can be produced by irradiation of either bacteria or phage before adsorption of the phage particles to the cells. Either the phage suspension or the bacterial suspension was exposed to light of saturating intensity, and the other was added within one second after turning off the light by discharging it rapidly into the irradiation tube from a serological pipette. The bacterial concentration was greater than $1x10^9$ cells per ml, so as to promote the most rapid possible adsorption. The temperature was 36.5° C. The plaque counts were not significantly different from controls receiving no light exposure.

However, when the phage and bacteria are first mixed, and a short light flash of saturating intensity is given the adsorption mixture at some stated time <u>after</u> the mixing, it is found that the ability to photoreactivate develops without perceptible delay from the time of mixing. Results of such an experiment are shown in figure 40. As before, the bacterial concentration was about $1x10^9$ per ml, and the temperature was 36.5° C. Mixing occurred at time t = 0, and the sample received a 5-second flash of intensity I = 1.0, starting at time t. The points plotted at t = 0 are controls which received no light.

Figure 41 shows results from a similar experiment in which the development of the ability to photoreactivate was

followed to completion. This required about 8 minutes and appeared to proceed approximately exponentially without lag. The time constant was about 1 3/4 minutes at the temperature 36.5° C. The bacterial concentration was about 3×10^9 per ml.

The reaction between phage and bacterium which these experiments show to be necessary before PhR can occur, may take place almost immediately following mixing, but on the average requires 1 3/4 minutes for completion at this temperature. It is not identical with the dark reactions previously described because the rates are not the same. It is not the first attachment of the phage particles to the bacteria, for this occurs almost instantaneously under the experimental conditions, as shown by the following results.

In the experiment of figure 41 potent antiserum against T2 was added to samples at 30, 60, and 90 seconds after mixing of phage and bacteria. (K value of serum = 1000 min^{-1}; dilution in sample 1:10. Therefore survival of free phage should be less than 1% within three seconds.) The samples were exposed to light 8 minutes after the initial mixing, and the PhR produced was identical with that in similar samples without serum. Therefore antiserum added after time 30 seconds does not prevent completion of the PhR-enabling reaction, although it would remove any free phage still present.

Heat-killed bacteria (65° C for 15 minutes) adsorb phage

T2 well and can also be used as an agent to remove free phage, since they do not liberate the phage and do not form plaques. In another experiment at 37°C, with a bacterial concentration of 2×10^9 per ml, phage and bacteria were mixed, and samples removed after a time t and diluted in a large excess of heat-killed bacteria. At t = 0, i.e. mixing in the presence of heat-killed bacteria, adsorption to healthy cells was effectively prevented, but at t = 30 seconds, the time of the first sample, this adsorption was already complete. The PhR-enabling reaction is therefore not the first adsorption step.

Garen and Puck (25) have studied the adsorption of T2 to bacteria under various ionic conditions. They found that attachment is irreversible with the buffered saline solution and the conditions we have used, but that at lower ionic concentrations it is reversible, and at still lower concentrations it does not occur at all. Dulbecco (26) has found that ionic conditions allowing only reversible attachment do not permit PhR. Puck has recently described (27) an experiment in which the adsorption mixture was first equilibrated under conditions permitting only reversible attachment, the ionic concentration then increased, and the development of irreversible attachment followed as a function of time. The time for half completion of the reaction was about one minute, or somewhat longer, which corresponds well with the time for the reaction permitting PhR.

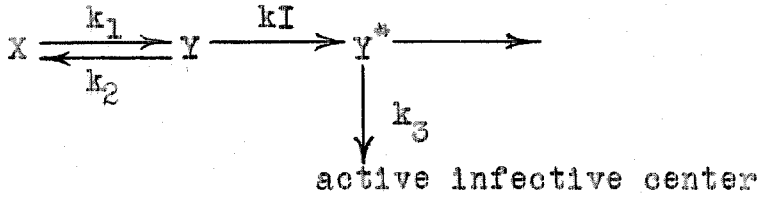
We interpret our results, therefore, to mean that in our buffer solution adsorption takes place in two sequential steps, the first a reversible attachment of the phage particle to the bacterial cell which is completed very rapidly at high bacterial concentrations, and the second a step of irreversible association of particle and cell which makes PhR possible, and which is the rate-limiting step in the development of the ability to photoreactivate. We conjecture further that this second step may be identical with the injection of DNA into the bacterium, as found by Hershey and Chase (11). It will be of great interest to see if additional work confirms this guess, and if more can be learned of the point at which the ultraviolet-damaged DNA enters into the PhR reaction scheme.

IV. Theory

The experimental results are very well in accord with predictions based on a simple model for the reaction mechanism. It would not be justified to assume that the model necessarily corresponds to the actual reaction mechanism of PhR, since many models can be proposed which will account for any given set of observations within the limits of experimental error. However, consideration of the model provides a useful guide to thinking and to experimentation because it clarifies the functional relationships between the experimental quantities.

The model we shall discuss is the following. In a bacterial cell infected by an ultraviolet-irradiated phage particle there is, in the dark, a temperature-dependent equilibrium between two compounds, X and Y. The reaction between X and Y follows first-order kinetics, with rate constants k_1 and k_2 for the forward and backward steps. Compound Y is capable of absorbing light and is converted by light to an active material, Y^*, which ordinarily disappears along unknown paths without effect. Y^* may, however, cause the phage to become active; the probability of reactivation per molecule of Y^* produced is k_3. X is assumed to be present in sufficient amount that it does not become appreciably depleted during the course of an experiment;

it is thus the reservoir of material from which Y is replenished. It is possible that Y^* may be cycled back to X either directly or indirectly. In summary:



We shall designate the amount of X and Y present as x and y and the probability that any phage particle of the photoreactivatable class is active as p. Then the complete expression for PhR produced during a single period of illumination of length t at constant intensity I can be found in a straightforward manner:

$$\frac{dy}{dt} = k_1 x - (kI+k_2)y \tag{7}$$

$$y = k_1 x\left(\frac{1}{kI+k_2}\right)\left[1 + \left(\frac{kI}{k_2}\right)e^{-(kI+k_2)t}\right] \tag{8}$$

$$\frac{dp}{dt} = k_3 \cdot kIy(1-p) \tag{9}$$

$$\ln(1-p) = -k_3 \cdot \int kIydt \tag{10}$$

$$= -k_3 \cdot k_1 x\left(\frac{kI}{kI+k_2}\right)\left[t + \left(\frac{1}{k_2}\right)\left(\frac{kI}{kI+k_2}\right)\left(1-e^{-(kI+k_2)t}\right)\right] \tag{11}$$

Equation (8) shows that y decreases from the dark equilibrium value of (k_1x/k_2) to a steady-state value of $(k_1x/kI+k_2)$. In the darkness following the light exposure y returns exponentially to the original equilibrium value

with time constant $(1/k_2)$:

$$\frac{dy}{dt} = k_1 x - k_2 y \tag{12}$$

$$y = \left(\frac{k_1 x}{k_2}\right)\left[1 - \left(\frac{kI}{kI+k_2}\right)e^{-k_2 t_D}\right] \tag{13a}$$

$$= \left(\frac{k_1 x}{k_2}\right)\left(\frac{kI}{kI+k_2}\right)\left(1 - e^{-k_2 t_D}\right) + \left(\frac{kI}{kI+k_2}\right) \tag{13b}$$

If a second short flash of light occurs at time t_D, its effectiveness will be proportional to the value of y at that time, i.e. to the value given by equation (13).

The correspondence of the theoretical with the experimental results is seen by comparing equation (11) with equations (1b), (2), and (4a), and with figures 3-31, and by comparing equation (13b) with equation (6) and with figures 32-38.

The expression (1-p) corresponds to the experimental value $(1 - \Delta P/\Delta P_{max})$. Equation (11) gives ln(1-p) as the sum of two terms, one of which is directly proportional to t and is much larger than the other term for all large values of t. In this case equation (11) corresponds to equation (1b).

In experiments where t is small, p is small also, and we have approximately, $-\ln(1-p) = p$. Then p is conveniently compared directly with ΔP, the increase in the experimental plaque count. With this approximation, equation (11) is seen to be identical in form with equation (2). The two equations correspond exactly if we equate the following

quantities:

$$R = k_3 \cdot k_1 x \left(\frac{kI}{kI+k_2}\right) \tag{14}$$

$$\Delta P_1 = R \cdot \left(\frac{1}{k_2}\right)\left(\frac{kI}{kI+k_2}\right) \tag{15}$$

$$A = (kI+k_2) \ . \tag{16}$$

The steady-state rate, R, is thus of the form given in equation (4b), and the identification is completed by equating

$$R_{max} = k_3 \cdot k_1 x \tag{17}$$

$$B = (k_2/k) \ . \tag{18}$$

Finally, a comparison of equations (6) and (13b) shows the agreement of the model with the results of experiments using intermittent light. The corresponding rate constants are

$$C = k_2 \tag{19}$$

The dependence on the absolute temperature, T, of the rate constant, k, of a single reaction step is given by the Arrhenius equation:

$$k = \text{Constant} \cdot e^{\frac{-\mu}{RT}} \ , \tag{20}$$

where μ is the energy of activation of the step, and R is the gas constant. A plot of ln k <u>vs</u>. (1/T) then gives a straight line of slope $(-\mu/R)$. The product or quotient of such rate constants also gives a straight line in this type of plot.

The model we have discussed makes the following identifications:

$$R_{max} = k_3 \cdot k_1 x \tag{17}$$

$$B = (k_2/k) \tag{18}$$

$$C = k_2 . \tag{19}$$

Each of these quantities should therefore give a straight line in an Arrhenius plot. Moreover, since k is a light reaction rate constant and is expected to change very little with temperature, both B and C should vary with temperature in the same manner. Figure 42 shows the data of tables 1, 2, and 3 plotted as suggested.

The experimental values of both B and C fit a single straight line rather well. The line drawn in figure 42 has a slope corresponding to μ = 11,600 calories per mole. The greater deviations of the data from the line at low temperatures are probably not significant, since the determinations are relatively quite inaccurate at lower temperatures, as can be seen by reference to figures 20, 21, 37, and 38.

Since B is measured in arbitrary units relative to the standard light intensity, the values of B could be shifted vertically on the log scale in figure 42 for purposes of comparison with the values of C. However B and C are approximately equal numerically without adjustment, indicating that the light intensity scale has been chosen accidentally such that the light reaction rate constant k = C/B = 1,

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$(1/k_2) = (1/C)$, a quantity already known from other experiments. Reference to figures 7-30 shows that ΔP_1 falls somewhat short of the expected value at the higher temperatures, but approaches it closely at low temperatures, where the light intensity is very large relative to the value of B. In experiments at the higher temperatures with repeated light flashes, such as that of figure 31, the value of ΔP_1 for the second and succeeding flashes has been found equal to that expected. The reduced size of ΔP_1 for the first flash in these cases is therefore another manifestation of the short initial lag in the beginning of PhR, which has been described.

V. Discussion

Our experiments provide considerable information of a formal nature on the mechanism by which light energy is coupled into the process of PhR. The importance of such information lies in the help it gives to identifying the substances involved and understanding their chemistry. It is of interest, therefore, to discuss in this relation what is known of the photochemistry of compounds of possible importance in PhR.

The first concern is the identification of the pigment. It is probably not present in free phage. Dulbecco (22) found no significant light absorption at the wave lengths of interest in free phage, either before or after ultraviolet irradiation. The action spectrum for PhR of Escherichia coli, B/r, found by Kelner (28) is so similar to that for PhR of bacteriophage T2 that it seems safe to assume that the chromophore is the same in both cases and is therefore of purely bacterial origin.

Kelner investigated the region from 365 mμ to much longer wave lengths using narrow bands isolated by means of interference filters from the continuous spectrum emitted by a tungsten lamp; isolated lines from the mercury spectrum were used for a few points. Maximum activity was found at 375 mμ, with measurable activity at wave lengths as long as

476 mμ. The exact shape of the curve is open to some question due to the failure of the reciprocity of time and intensity which he found. The direction of the effect was such as to lead to underestimation of the activity of wave lengths giving low rates of reactivation. (This was probably due to the lack of stability of the preparations during long exposures, an effect described both by Kelner (29) and by Novick and Szilard (30), who also worked with PhR of Escherichia coli, B/r.) However the position of the maximum and the general extent of the curve are probably correct. Because of the importance of the curve to the discussion at hand, Kelner's data are reproduced in figure 43.

We know of three types of compounds of possible interest which have an absorption maximum near 375 mμ. Pyridoxal phosphate has a peak at about 385 mμ, with absorption extending to about 450 mμ on the long wave length side (31). This fits the PhR action spectrum rather well, but the possible function of pyridoxal phosphate in PhR would be completely unknown. The pteridines show an absorption maximum at 365-375 mμ. Pteroylglutamic acid has an absorption maximum at 365 m , but absorption extending only to about 410-420 mμ (32), which is scarcely a long enough wave length to agree with the PhR action spectrum.

The flavins seem the most likely candidates for identification as the PhR chromophore. They have an absorption

maximum at 370-385 mμ, which corresponds very well with the PhR action spectrum, and a second equally strong maximum at 455 mμ (33). Kelner rejects them because of the absence of this second maximum in the action spectrum. However, after examining Kelner's data and replotting them (figure 43), we believe that there is evidence of considerable PhR in the longer wave length region, and that this may indicate that the second flavin absorption peak is present but leads to PhR with reduced quantum efficiency. There is no necessity for supposing the quantum yield to be the same at all wave lengths, and even the complete absence of PhR in the longer wave length region would not constitute sufficient objection to exclude the flavins. (Loufbourow (34) discusses the assumptions involved in the use of action spectra to identify pigments.)

Of importance in the discussion of the possible PhR pigment is the absolute rate of the light reaction step, which has been measured in our experiments. This can be compared with the rate of light absorption, expressed in quanta per pigment molecule per second, calculated from the incident light intensity and the known absorption coefficient of the assumed chromophore. The comparison shows that the light reaction proceeds with very high quantum efficiency, apparently almost unity, and that the rate of the reaction can be accounted for only by a chromophore with an absorption coefficient comparable to that of the flavins.

The absorption coefficient of the flavins at the short wave length maximum is approximately $\beta = 2.4 \times 10^7$ cm^2 per mole (33). (β is defined by the relation, $I/I_0 = e^{-\beta cd}$, where c = concentration in moles per cm^3, d = path length in cm.) Expressed as a molecular cross section this is $(\beta / N_0) = (2.4 \times 10^7)/(6.0 \times 10^{23}) = 0.4 \times 10^{-16}$ cm^2 per molecule.

The total incident light energy and the energy measured through the 379 mμ filter were approximately 2.5 and 0.025 calories per cm^2 per minute, respectively. As an estimate based on these values, and on the transmission of the filter, the spectral distribution of the energy indicated by measurements through other filters, and the width of the action spectrum for PhR, it seems reasonable to assume that a total energy equivalent to not more than about 0.3 calorieper cm^2 per minute at the 379 mμ peak is available in the light beam. This equals 4×10^{16} quanta (380 mμ) per cm^2 per second.

Therefore at this light intensity there would be about $(0.4 \times 10^{-16})(4 \times 10^{16}) = 1.6$ quanta absorbed per flavin molecule per second. The observed rate of the light reaction step at this intensity at 0° C was $(1/0.6) = 1.7$ per pigment molecule per second. In other words, the light intensity is just sufficient to produce the observed reaction rate if the chromophore is a flavin and if the reaction proceeds with a quantum efficiency of unity.

The absorption coefficients of the other possible chromophores are lower: for pyridoxal phosphate, $\beta = 1.0 \times 10^7$ cm^2 per mole, and for the pteridines, $\beta = 1.5 \times 10^7$ cm^2 per mole, approximately, at the absorption maximum. In order to account for the observed reaction rate on the basis of either of these it would be necessary to revise the estimate of the intensity upward considerably. This possibility cannot be excluded because of the crude nature of the intensity measurement, but it seems unlikely. Experiments with monochromatic light of accurately known intensity are urgently needed. An action spectrum giving the absolute rate of the PhR light reaction as a function of wave length could now be obtained with considerable accuracy, and would probably give much more definite information on the identity of the chromophore as well as further information necessary for understanding the nature of the light reaction step.

Known photochemistry involving the flavins includes several examples of reactions photosensitized by riboflavin (35) and the photochemical decomposition of riboflavin itself (36), but none of these appears to be of immediate importance in PhR. Of interest, however, is the quenching of the fluorescence of riboflavin and of flavin-adenine-dinucleotide (FAD) by purines and pyrimidines, which has been studied by Weber (37). Working principally with caffeine and adenine (both purines), he concluded that a

complex is formed between the purine and flavin which does not fluoresce, although its light absorption is not appreciably different from that of the free flavin. The mean life of the complex could only be stated to be long (greater than 10^{-6} second) compared to the mean life of the excited state (1×10^{-8} second) of the flavin. The complex becomes more dissociated as the temperature is increased; the heat of dissociation is 1.6 ± 0.6 kilocalories per mole.

The quenching of riboflavin fluorescence can be observed easily by eye without special preparations; it occurs with purines, with pyrimidines, and with nucleic acid. We have noticed that both the association and dissociation reactions proceed at least as fast as the mixing of solutions can be accomplished in a test tube (room temperature). Therefore this reaction cannot be identified with the much slower rate-limiting reactions we have found in PhR; but neither can it be excluded from the process, since it would be completed too rapidly to be noticed. This illustrates both the usefulness and the limitation of the experiments we have performed. It is possible that a similar reaction involving a different flavin compound or, particularly, pyrimidines altered by ultraviolet radiation may be found to have characteristics identifiable with those of the PhR reactions. However, the essential point is that flavins do combine with purines, and presumably with pyrimidines also, and that the disposition of light energy received by the flavin is

thereby altered in a very effective manner.

Sinsheimer (38) has discovered that the alteration of certain pyrimidine compounds by ultraviolet light is partially reversible under some conditions. This takes place with uracil, with uridine, and with cytidylic acid; cytosine, but not uracil, is ordinarily found in DNA. Uracil, in solution at pH 7, was exposed to ultraviolet radiation (230-280 mμ), and "decomposition" was measured spectrophotometrically by the decrease in absorption at the 259 mμ absorption maximum of uracil. Change of the pH to 1.0 brought about recovery of absorption with first-order kinetics and a time constant of 14 minutes at room temperature; the recovered material appeared to be uracil. Light absorption at 259 mμ was first reduced by the ultraviolet irradiation to 37%, then recovered to 74% of the original value. Similar recovery was produced at pH 7 by raising the temperature to 100°C, but the results were less reproducible. Uridine behaved similarly, but was more sensitive to ultraviolet radiation; results with cytidylic acid were described as preliminary. Under the given conditions thymine, cytosine, adenine, guanine, adenylic acid, and guanylic acid were not decomposed.

It is possible that partial recovery of pyrimidines from ultraviolet radiation damage might also be produced at neutral pH and at normal temperatures if some means other than heat were found to supply energy to the pyrimidine, and that this might be accomplished by light through the mediation

of a flavin pigment. This may constitute the essential mechanism of PhR.

This conjecture raises the question whether direct repair of the ultraviolet radiation damage is compatible with the kinetics of PhR. There are almost certainly numerous independent points of damage produced within the organism by the radiation. If these damages are repaired point by point, then the reactivation kinetics must be multiple-hit in character. That is, since at low survivals most individuals will have much more than a lethal amount of damage, a number of independent acts of repair must occur in most before activity is regained; initially the rate of appearance of active individuals will be quite low, but the rate will increase rapidly during the course of reactivation, leading to a large inflection at the beginning of the curve. In general this is what is actually found. Bacteriophage T2 is an exception, however.

PhR of T2 appears to follow first-order kinetics. Because of this it has been assumed that a single reactivating event is necessary to accomplish PhR, and therefore that PhR of T2 cannot consist of a direct repair of multiple damages. It has been suggested, as an explanation, that the action of the reactivating light is to by-pass some essential step normally performed by the nucleic acid but blocked in the presence of ultraviolet radiation damage, so that the characteristics of the reactivation are independent of the

number of damages present. However, we think it is unreasonable to suppose that a single quantum-event can accomplish a function for which a very large number of units are ordinarily essential. The kinetics of PhR of T2 are not perfectly first order, as has been pointed out, and we suggest that they can be explained on the basis of a direct repair mechanism. These points will now be discussed in more detail.

There are about 500,000 nucleotide units in the phage particle, and about 40,000 of these are so essential that the damage of any one is lethal (12). The ultraviolet survival curve of T2 is approximately exponential, but has a small initial inflection; therefore, at a survival of 10^{-3} there are an average of about 7, or more accurately about 10 lethal hits per particle. The survival curve after maximum PhR is very similar in shape, but has a reduced slope. It indicates that about half of the original hits can be reactivated. Since the quantum yield is about 0.00015, an average of 10 hits means that about 60,000-70,000 quanta have been absorbed per particle, or only one nucleotide in 7-8 has even received a light quantum. The 10 hits must therefore occur at 10 distinct points scattered more-or-less at random through the vital material of the particle. They cannot be thought of as successive events occurring at one essential locus and causing only one true point of damage.

Suppose now that there is some essential reaction step in phage reproduction which is normally performed by the

nucleic acid of the particle, but which is blocked in the presence of ultraviolet radiation damage. In order that damage to any one of some 40,000 nucleotide subunits of the nucleic acid can cause a lethal block it is necessary to suppose that every one of these units physically engages the mechanism of the reaction at some time before it is properly completed. If the reaction is completed as a single step in time, so that it could be by-passed in a single step, it must involve simultaneously all of the 40,000 nucleotides, which total more than one million in molecular weight. It seems impossible that a single light quantum could produce a reaction with highly specific effects extending throughout the enormous structure that would be required. It would be possible to suppose instead that the machinery of the essential step is small and involves all of the 40,000 nucleotides by reacting with them one at a time in sequence. By-pass of the blocked step could then be produced easily by a single quantum, but the by-pass would have to be repeated as each damaged point is reached. Reactivation of the particle as a whole could not be produced by a single event.

If PhR occurs by direct repair of multiple damages, then ordinarily the rate of reactivation should increase during the early stages. This effect is found in the PhR of T2, but it is so small that the over-all kinetics appear to be almost exactly first order, or one hit. We suggest that the PhR of T2 can nevertheless be explained on the basis of a

direct repair mechanism and that the simulation of first-order kinetics results from inhomogeneity of the damages with respect to ease of repair during PhR. Reactivation would occur rapidly at first due to recovery of the most easily repaired damages, but would soon slow down and approach the rate characteristic of the most slowly repaired damages. This decrease in the rate of reactivation counters the increase in rate otherwise expected in multiple-hit repair and can, under some conditions, lead to a close approximation to one-hit PhR. The necessary conditions are not critical. Sample calculations and results are shown in the appendix. The approximation becomes poorer at large ultraviolet doses, but experiments uncomplicated by multiplicity reactivation cannot then be performed. A suitable interpretation of multiplicity reactivation or of the interaction of PhR and multiplicity reactivation is not known. Circumstances thus confine all work to a narrow range. A variation among the damaged points of the rate of repair during PhR might arise from actual chemical differences in the nature of the damage, or from differences in the ease with which the points enter into the dark reactions of PhR due only to differences of their position within the structure of the nucleic acid.

Recognizing it as pure conjecture, we suggest the following over-all picture of PhR of bacteriophage T2. The principal lethal effect of ultraviolet irradiation is the

alteration of pyrimidines of the phage DNA. Following adsorption of the phage particle to a bacterial cell the pyrimidines combine reversibly with a flavin pigment of bacterial origin. Light energy absorbed by the flavin is then available, at least in part, to the pyrimidine, and this makes possible a partial recovery from the alteration produced by the ultraviolet radiation. Associated with the utilization of light energy are dark reactions, which have been demonstrated in our experiments. There is at present no basis for useful speculation as to the nature of these dark reactions, but knowledge of them provides the means of making a positive identification between chemistry which may be discovered in vitro and the in vivo process of PhR.

This conjectural scheme is useful because it suggests numerous possibilities for further work. It is interesting that it also provides a basis for explaining various features of PhR found in diverse organisms. Points that may be so explained include the following: 1.) PhR is of widespread occurrence; the suggested scheme involves only components generally present in all living things. 2.) PhR affects many types of manifestation of ultraviolet radiation damage (for example, see 39, 40, 41); this is easily understandable in terms of direct repair of one common type of damage. 3.) The effect of PhR is generally closely equivalent to the reduction of the ultraviolet dose by a constant factor independent of the dose (29, 30). The biological effects

of damage to various pyrimidines of the nucleic acid would be expected to be similar, so that the repair of a selected class of the damages by PhR would lead to effects very similar to those produced by a smaller ultraviolet dose without PhR; the relative sizes of the reactivatable and non-reactivatable classes should be independent of the dose, since no single point receives more than one hit at ordinary doses. 4.) In the case of Streptomyces griseus the PhR action spectrum seems to indicate that some pigment other than a flavin is used (28). We have ascribed a secondary role to the pigment, however; it is possible that the reaction between pyrimidines and flavins is not unique, and that another pigment, if present in large amount, would react similarly and serve to couple light energy into the process of PhR. 5.) Almost no PhR is possible following X-ray inactivation of phage (22). PhR has been pictured as a rather specific effect occurring only with a particular type of damage frequently produced by ultraviolet radiation; X-rays, in comparison, produce a wide variety of damages, most of which would not be expected to recover during PhR.

If PhR occurs by direct repair of the damage, then it leaves untouched the important question of why alteration of the nucleic acid is lethal. What is the essential role played by nucleic acid in reproduction? It is possible, however, that if ultraviolet light does prove to be rather specific in its effects, a more complete understanding of

these effects will make it a useful tool in further investigations.

VI. Summary

Detailed studies have been carried out of the kinetics of photoreactivation of bacteriophage T2r inactivated by ultraviolet light and adsorbed in buffered saline solution to washed cells of Escherichia coli, strain B. All results are for single infection. The criterion of activity is the ability to form a plaque when plated on agar by a standard technique. The following experimental results are found:

1.) The initial rate of reactivation is directly proportional to the light intensity and almost independent of the temperature. At high light intensities the rate decreases very rapidly, as an exponential function of time, to a lower value which thereafter remains constant. The rate of decay of the reactivation rate is approximately proportional to the intensity and is almost independent of the temperature.

2.) The steady-state rate, R, which prevails after the transient initial period, can be expressed as a function of intensity, I, as

$$R = R_{max} \left(\frac{I}{I+B}\right) ,$$

where R_{max} is a maximum, or limiting rate, and B is a constant. Both R_{max} and B are functions of

the temperature and have been measured in the range 0-45° C.

3.) During further continuous illumination the number of active infective centers increases with time approximately as the function $(1 - e^{-at})$, where a is a constant.

4.) If a long dark interval is allowed to follow a flash of intense light, the system completely recovers from the effects of the light, and a second flash produces the same amount of reactivation as the first. If the length of the dark interval, t_D, is varied, the progress of the dark reaction can be followed as a function of time. It is found to proceed as $(1 - e^{-Ct_D})$. The constant C changes with temperature and has been measured in the range 0-45° C.

5.) If the temperature is changed immediately after a light flash, the amount of reactivation obtained is unaltered. If the temperature is changed before the light flash, however, time is required for dark reactions to reach equilibrium, and the yield of reactivation from the flash depends on its timing relative to the temperature change.

6.) Experiments on the relation of photoreactivation to adsorption show that no reactivation is produced by exposure to light of either the bacteria

or the free phage particles before they are mixed, but at high bacterial concentrations the ability to be reactivated develops without observable lag from the time of mixing. It increases approximately exponentially, with a time constant of about 1 3/4 minutes at 37° C, reaches a maximum in about 8 minutes, and remains constant thereafter. The step required to permit photoreactivation is not the first attachment of the particles to the bacteria; it is probably the second, or irreversible step of adsorption described by Puck and Garen.

A simple theoretical model for the reaction mechanism is discussed. The experimental results can be accounted for on the basis of this model. It would not be justified, however, to assume that the model necessarily corresponds to the actual reaction mechanism of PhR.

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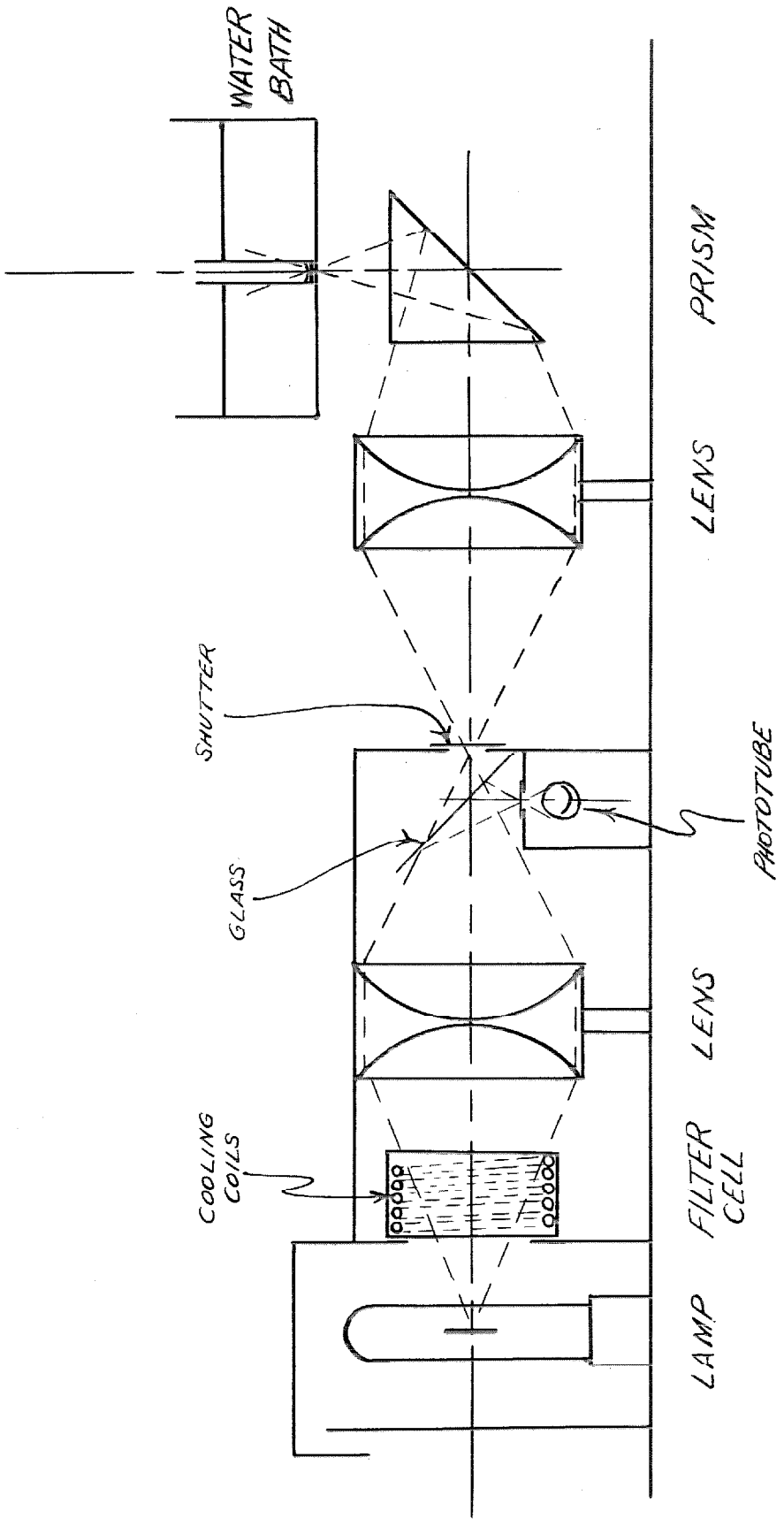
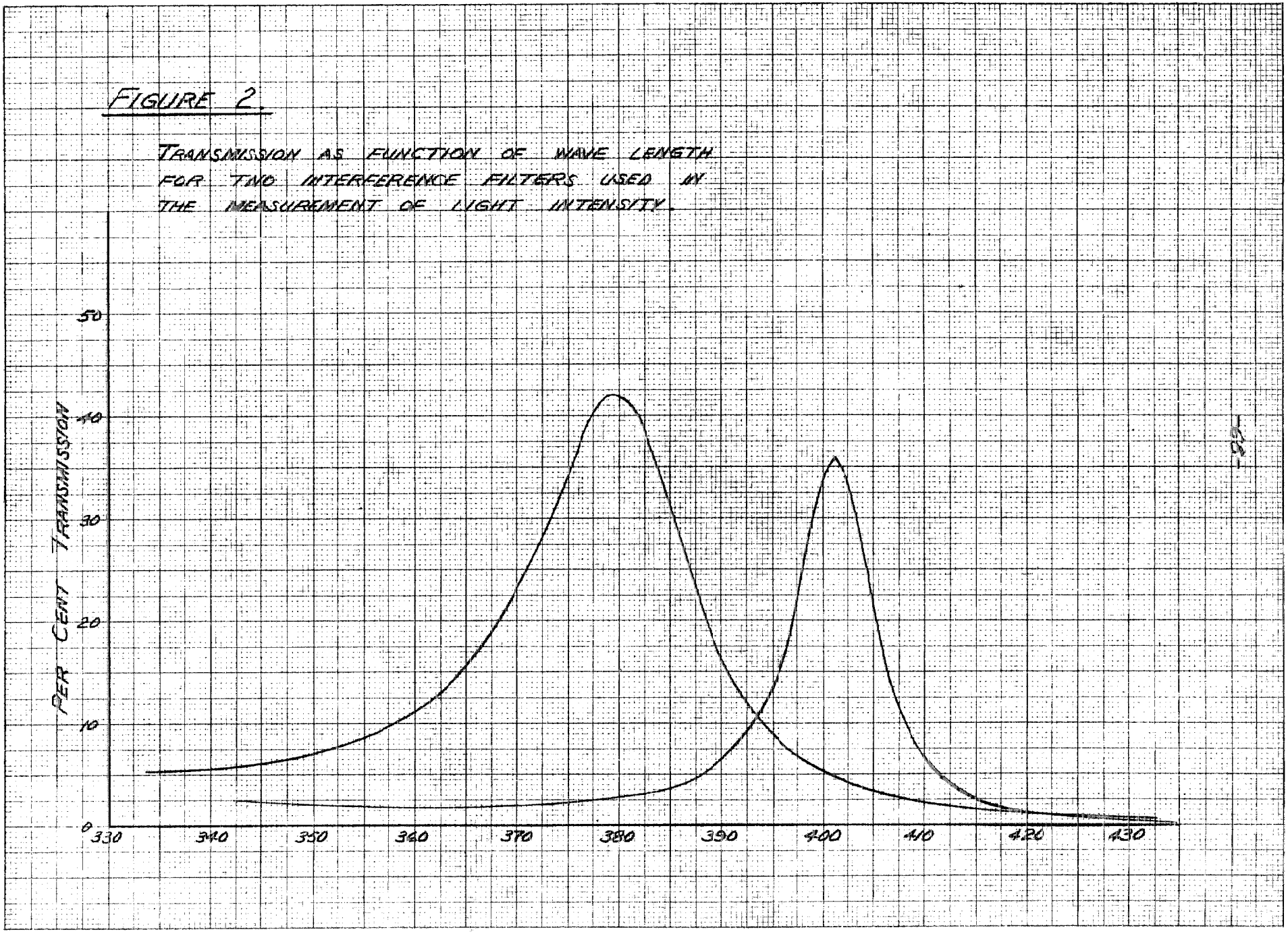


FIGURE 1: EQUIPMENT USED FOR PHOTOREACTIVATION.

FIGURE 2

TRANSMISSION AS FUNCTION OF WAVE LENGTH FOR TWO INTERFERENCE FILTERS USED IN THE MEASUREMENT OF LIGHT INTENSITY.



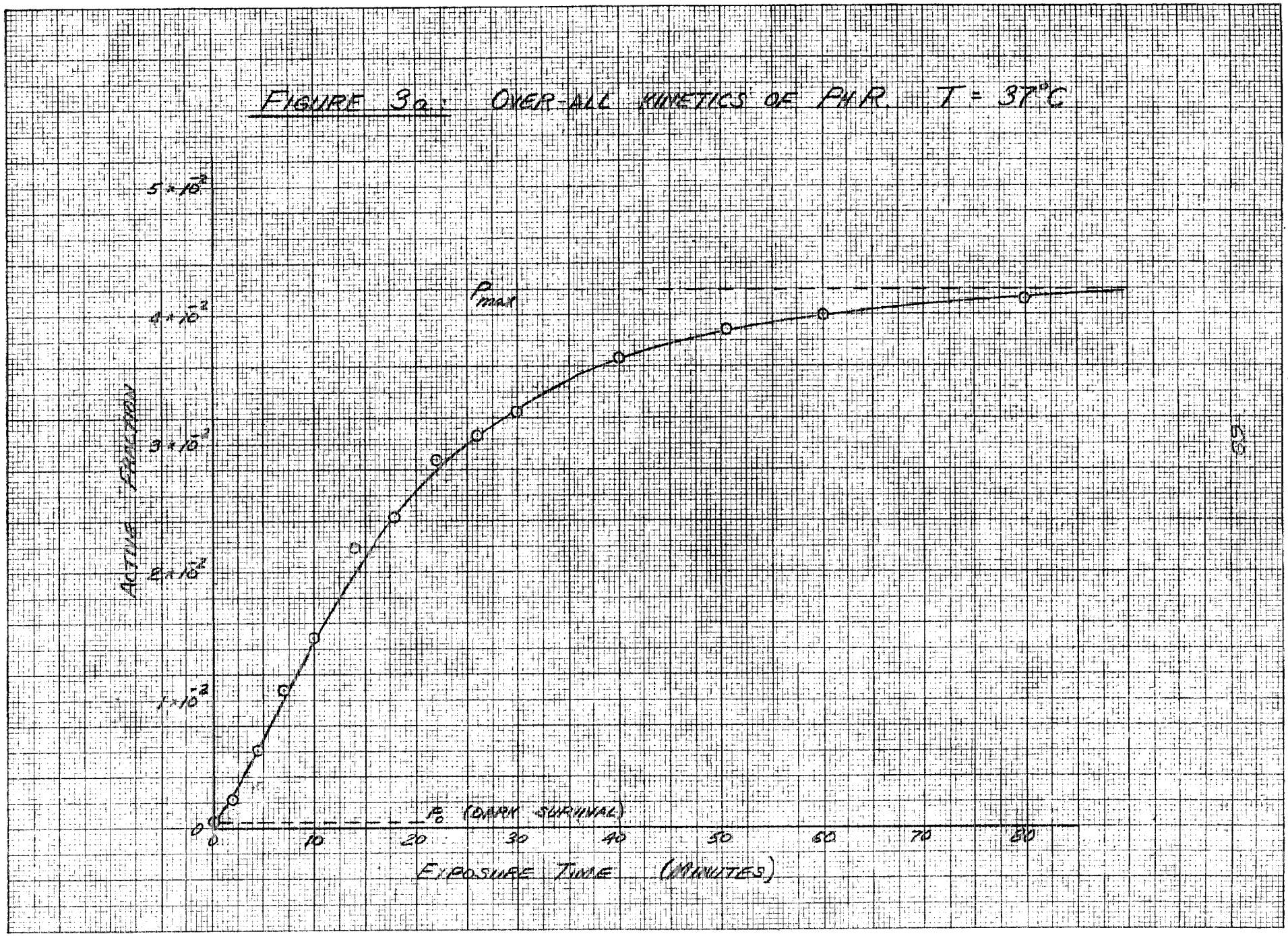


FIGURE 3a: OVER-ALL KINETICS OF PHR. T = 37°C
5×10^{-2}
4×10^{-2}
3×10^{-2}
2×10^{-2}
1×10^{-2}
0
P_{max}
P_0 (ZERO SURVIVAL)
ACTIVITY
0
10
20
30
40
50
60
70
80
EXPOSURE TIME (MINUTES)

FIGURE 3b.

SAME EXPERIMENT AS FIGURE 3a.

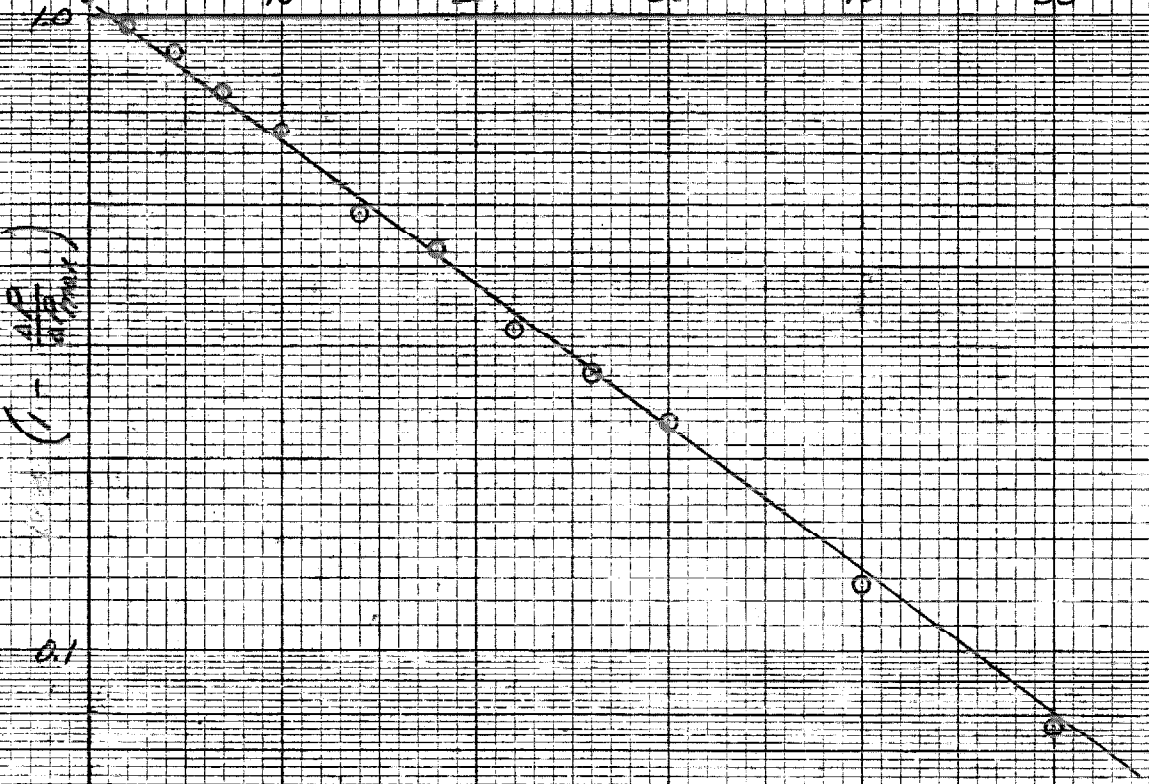


FIGURE 4a: TRANSIENT INITIAL PERIOD; $T = 37.0^{\circ}C$

4 LIGHT FLASHES OF LENGTH t, INTENSITY I AT 3-MINUTE INTERVALS.

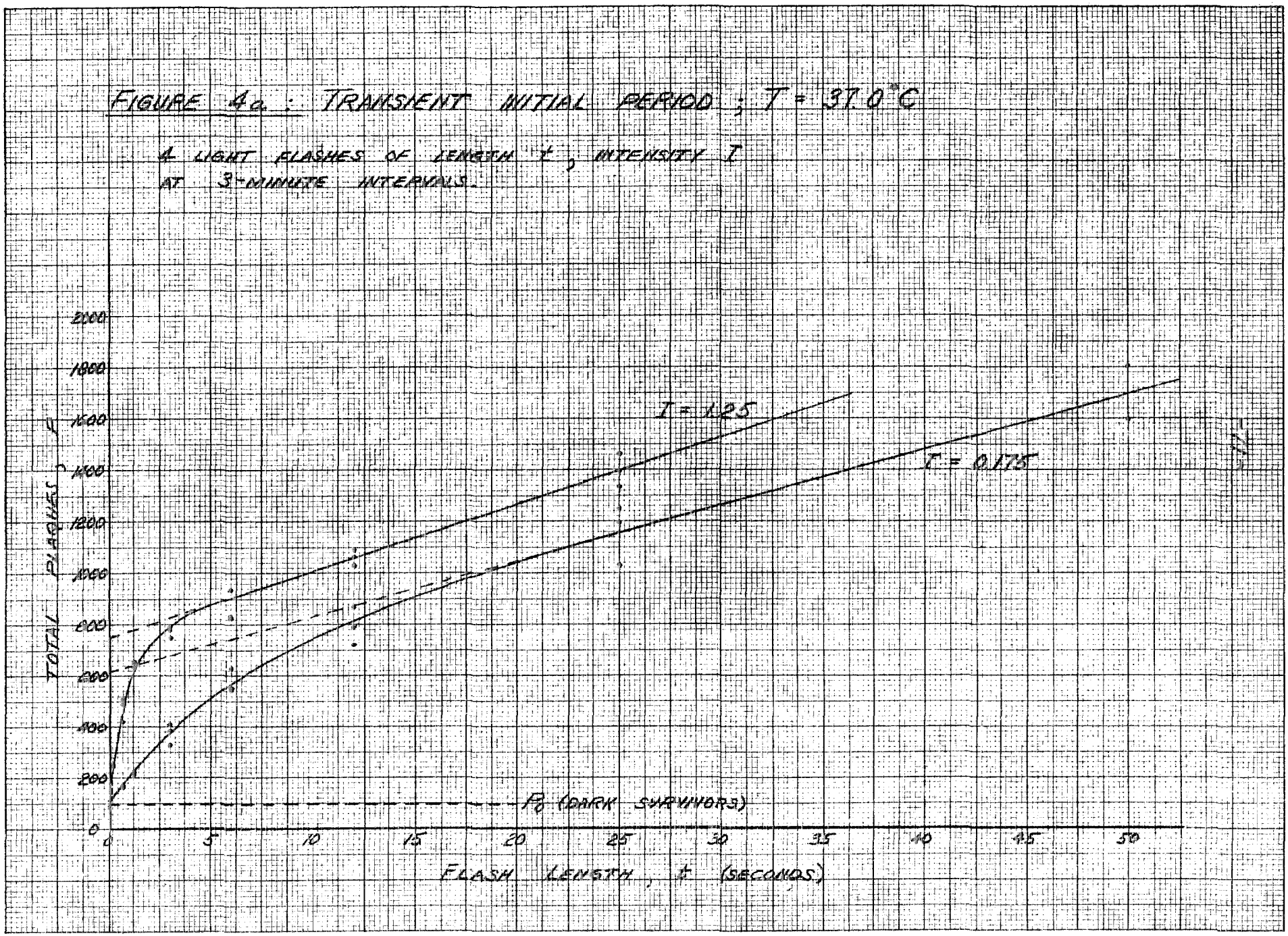
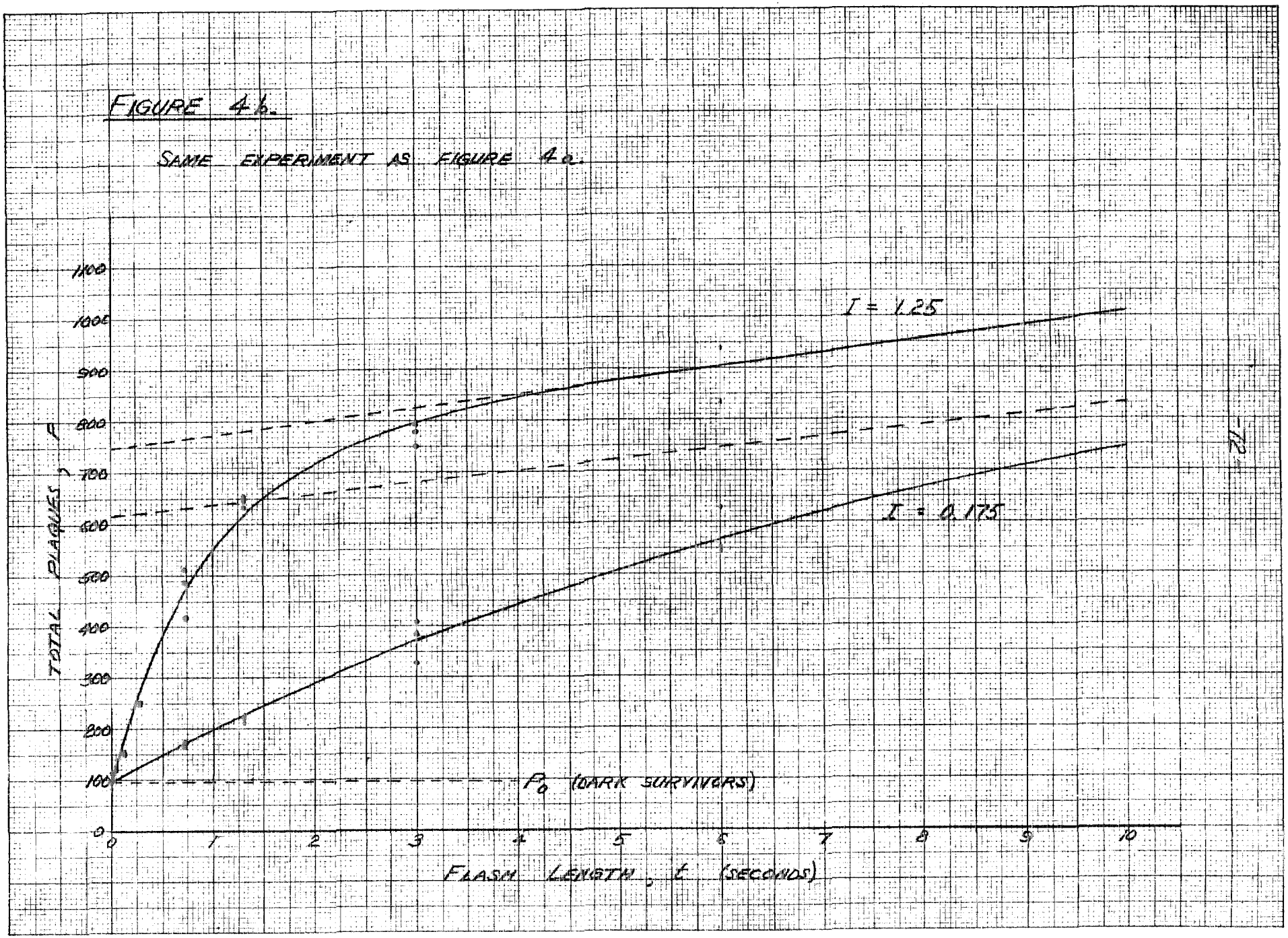


FIGURE 4b.

SAME EXPERIMENT AS FIGURE 4a.



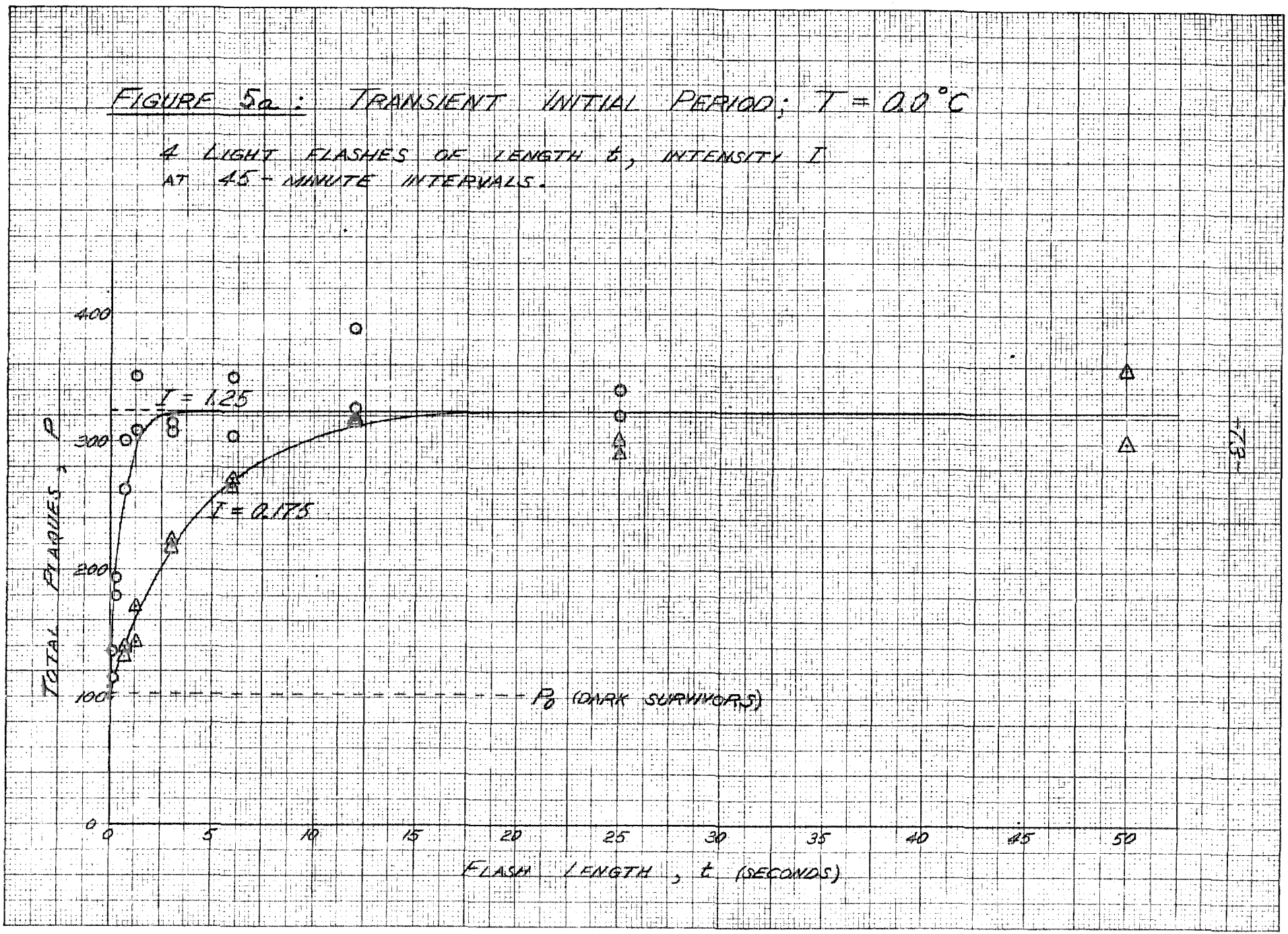


FIGURE 5a: TRANSIENT INITIAL PERIOD; T = 0.0 °C
4 LIGHT FLASHES OF LENGTH t, INTENSITY I
AT 45-MINUTE INTERVALS.
400
300
200
100
0
P
TOTAL PLAQUES
I = 1.25
I = 0.175
P0 (DARK SURVIVORS)
0
5
10
15
20
25
30
35
40
45
50
FLASH LENGTH, t (SECONDS)

FIGURE 5b: TRANSIENT INITIAL PERIOD; T = 0.0°C.

SAME EXPERIMENT AS FIGURE 5a.

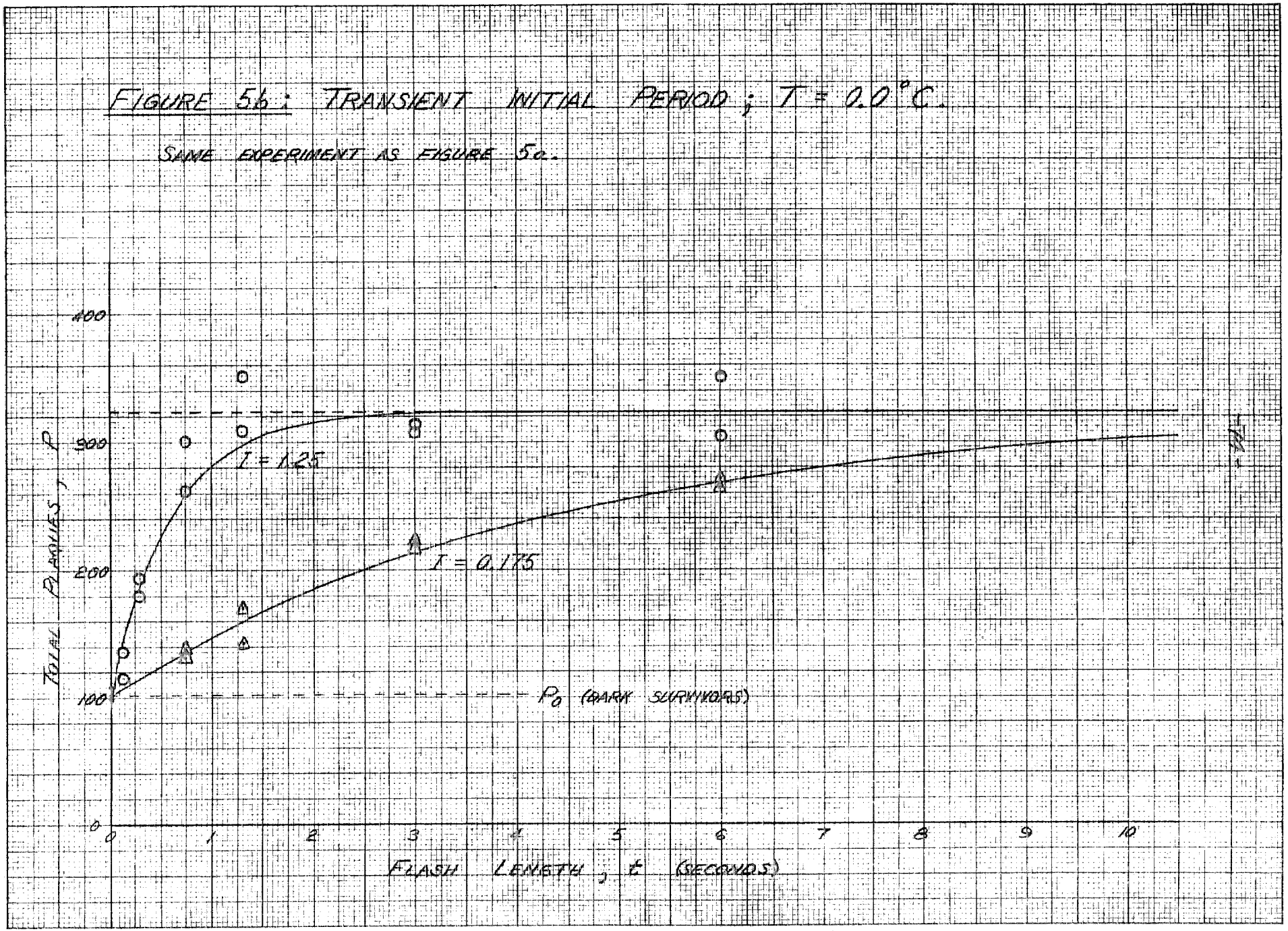


FIGURE 62: RELATION OF INITIAL PERIOD TO OVER-ALL PHP CURVE

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I = 0.6 ; T = 32.5 °C

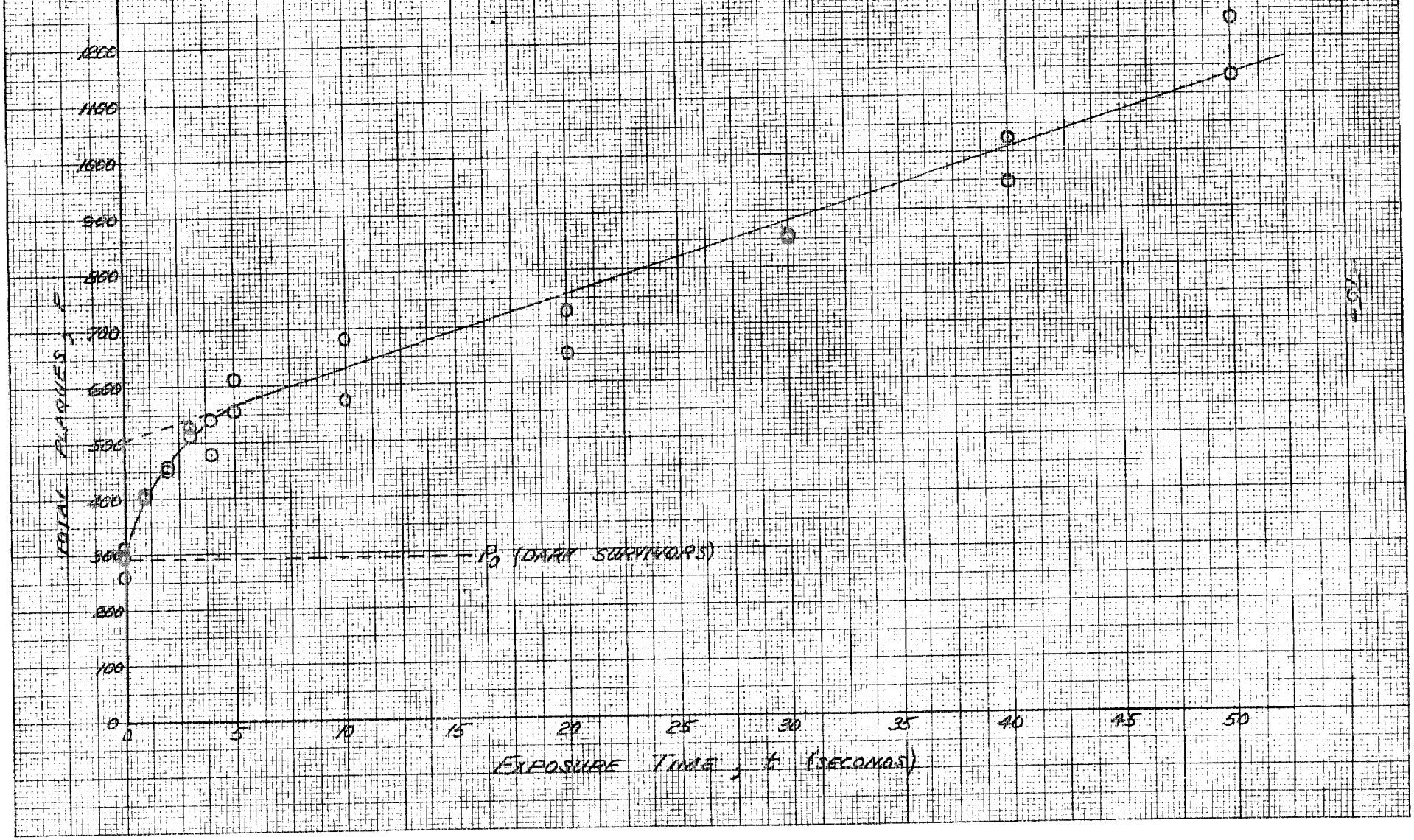


FIGURE 6b.

SAME EXPERIMENT AS FIGURE 6a.
ADSORPTION MIXTURE DILUTED BY FACTOR OF 10
BEFORE THESE SAMPLES PREPARED.

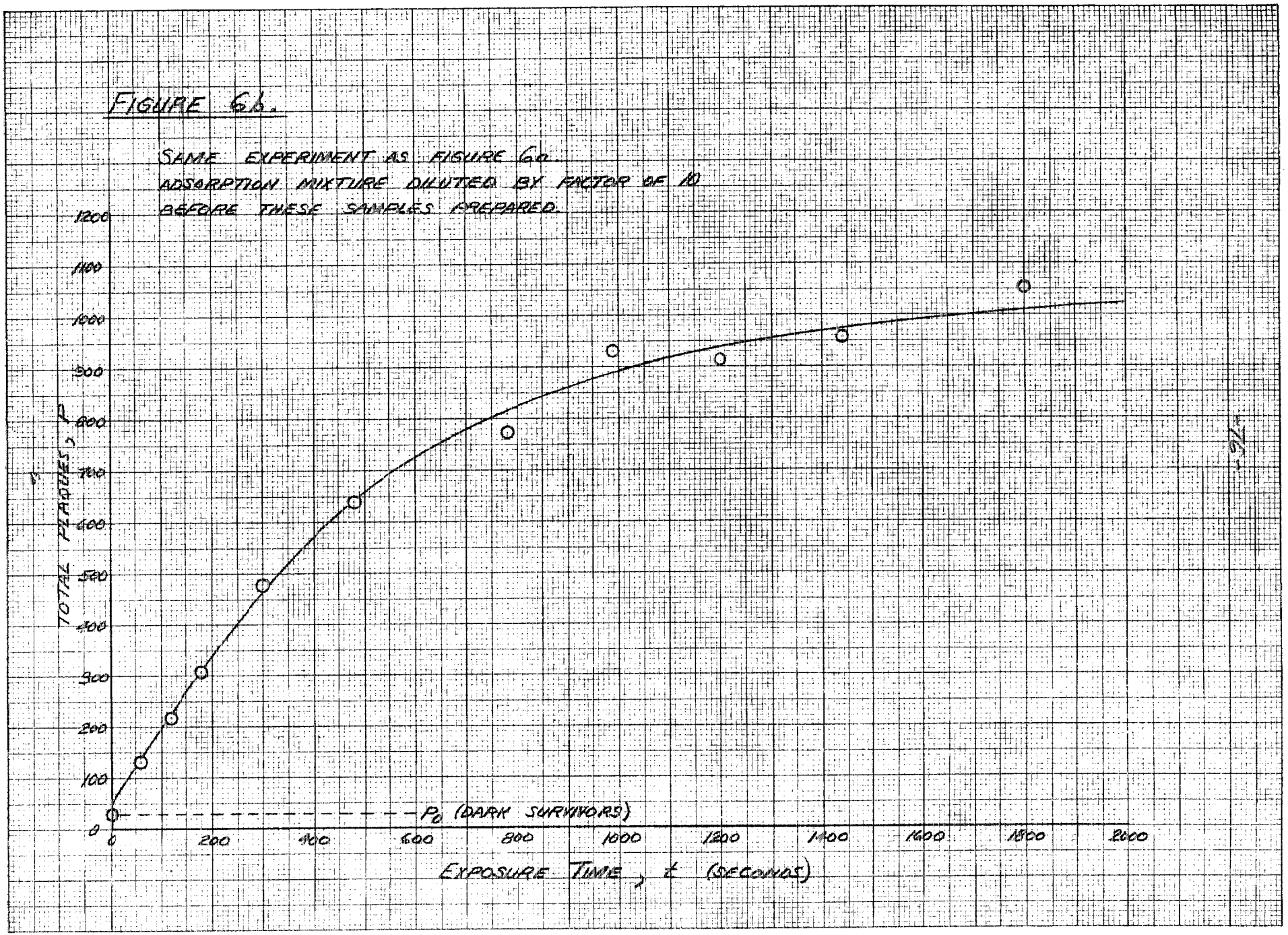
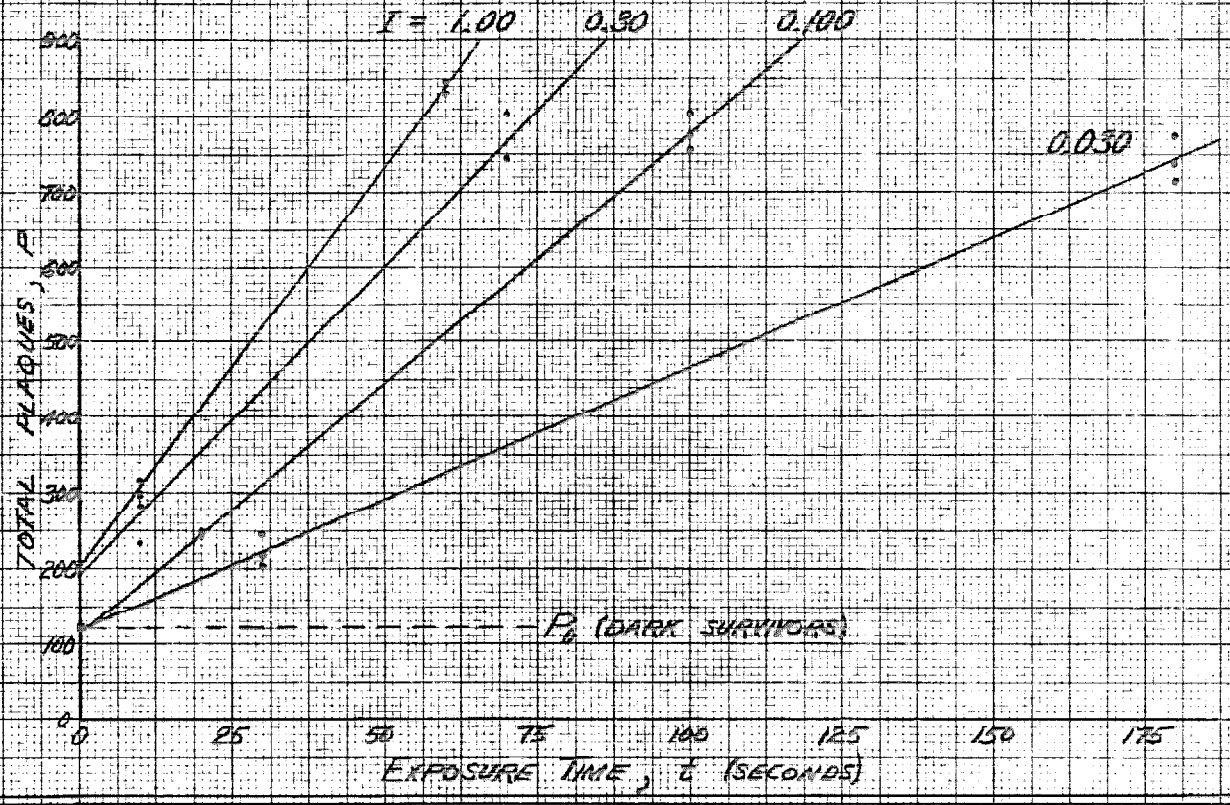


FIGURE 7: PHR RATE VS. LIGHT INTENSITY; T = 45.5°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I.



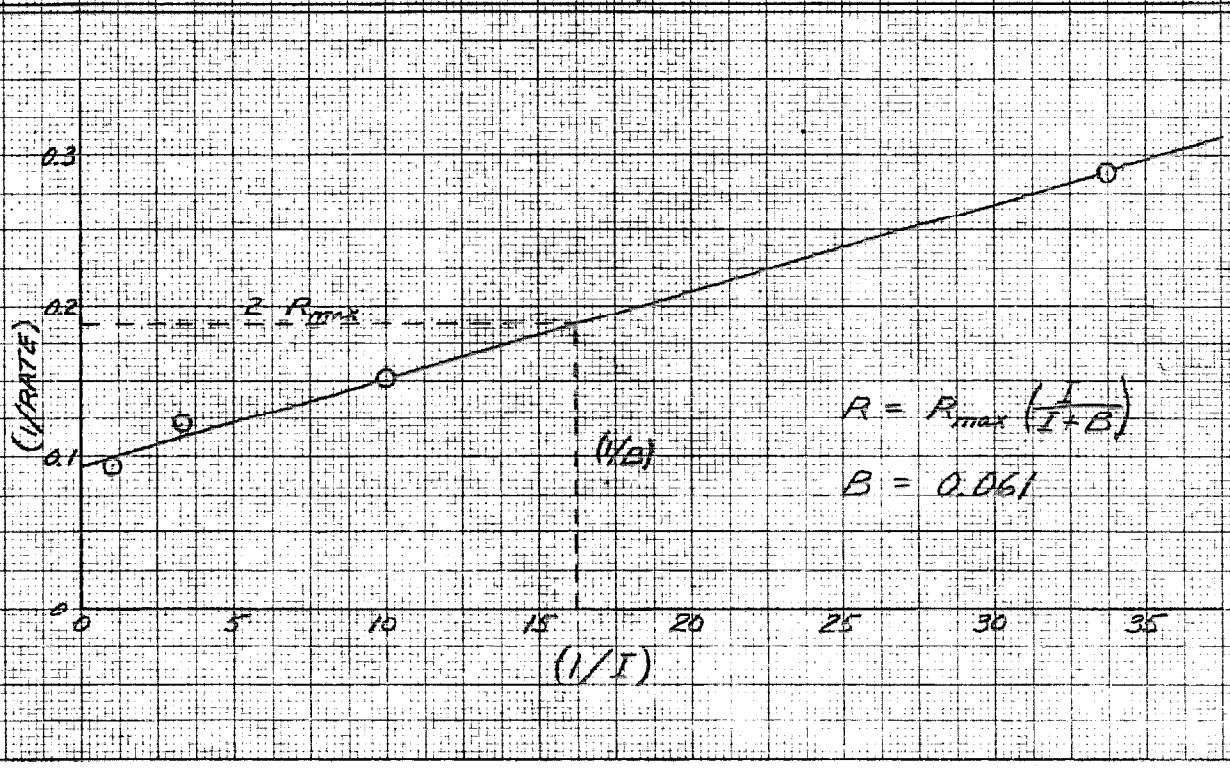
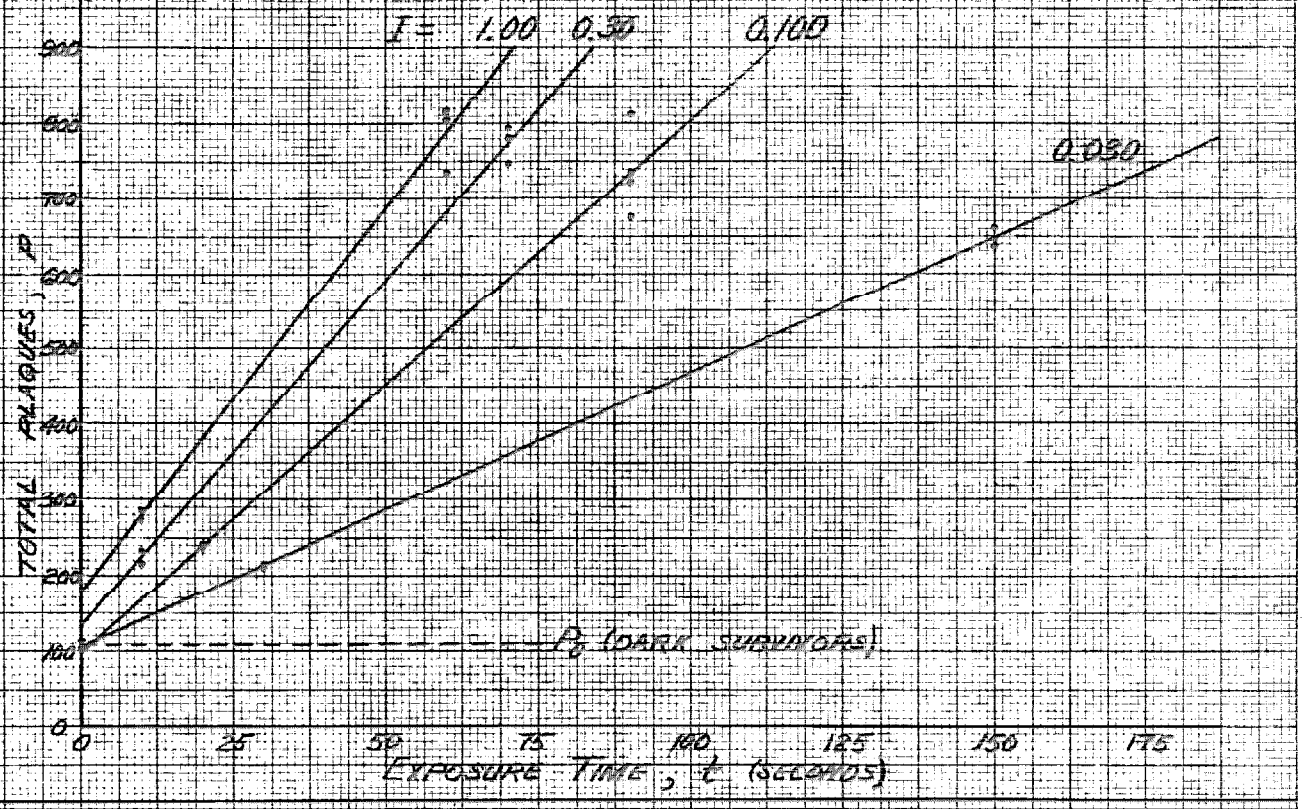


FIGURE 8: PHR RATE VS LIGHT INTENSITY; T = 45.4 °C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I



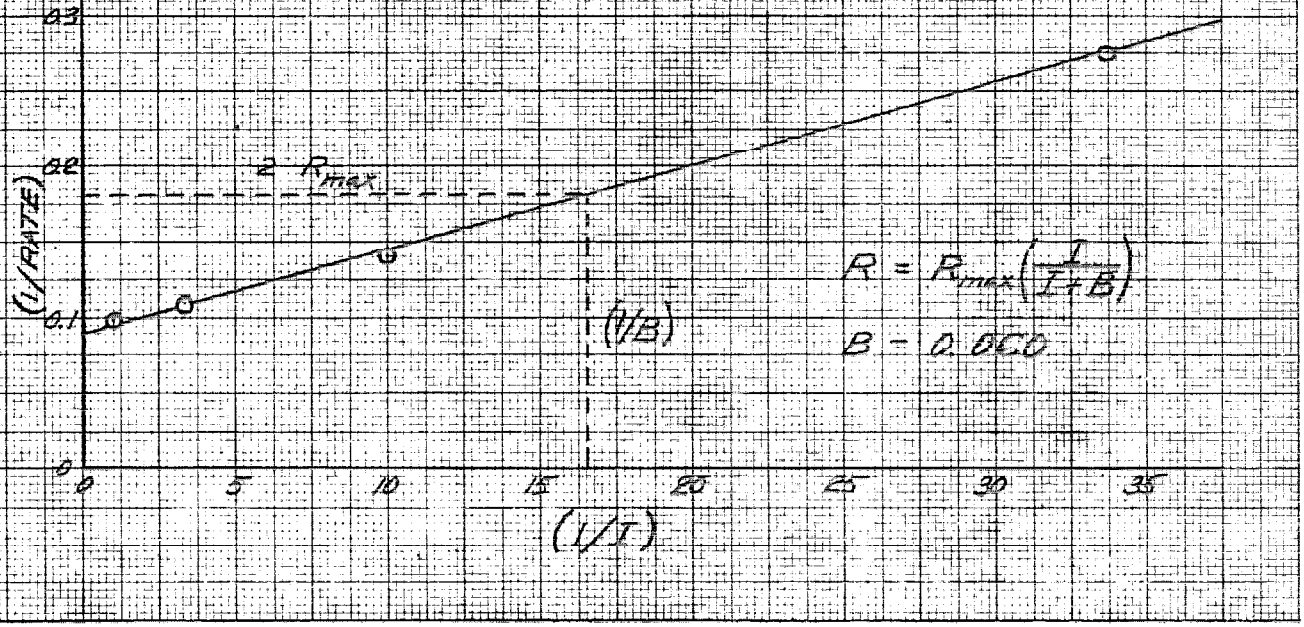
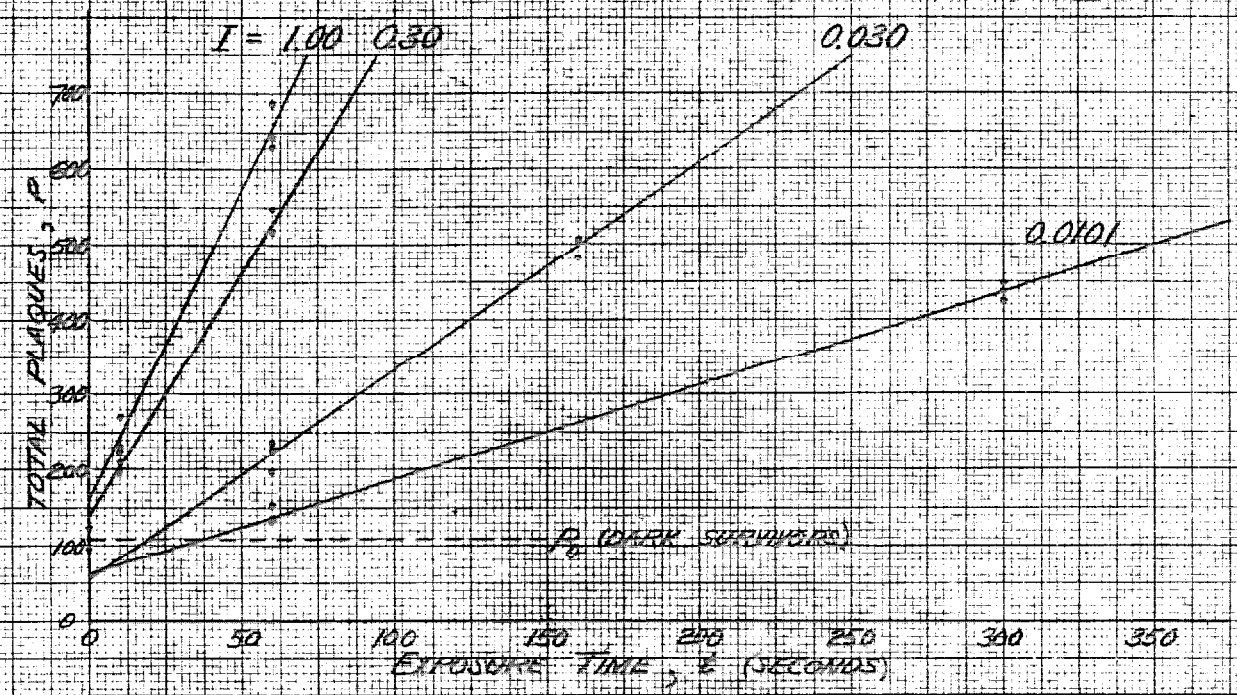


FIGURE 9. PHR RATE VS. LIGHT INTENSITY; T = 4.7°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I



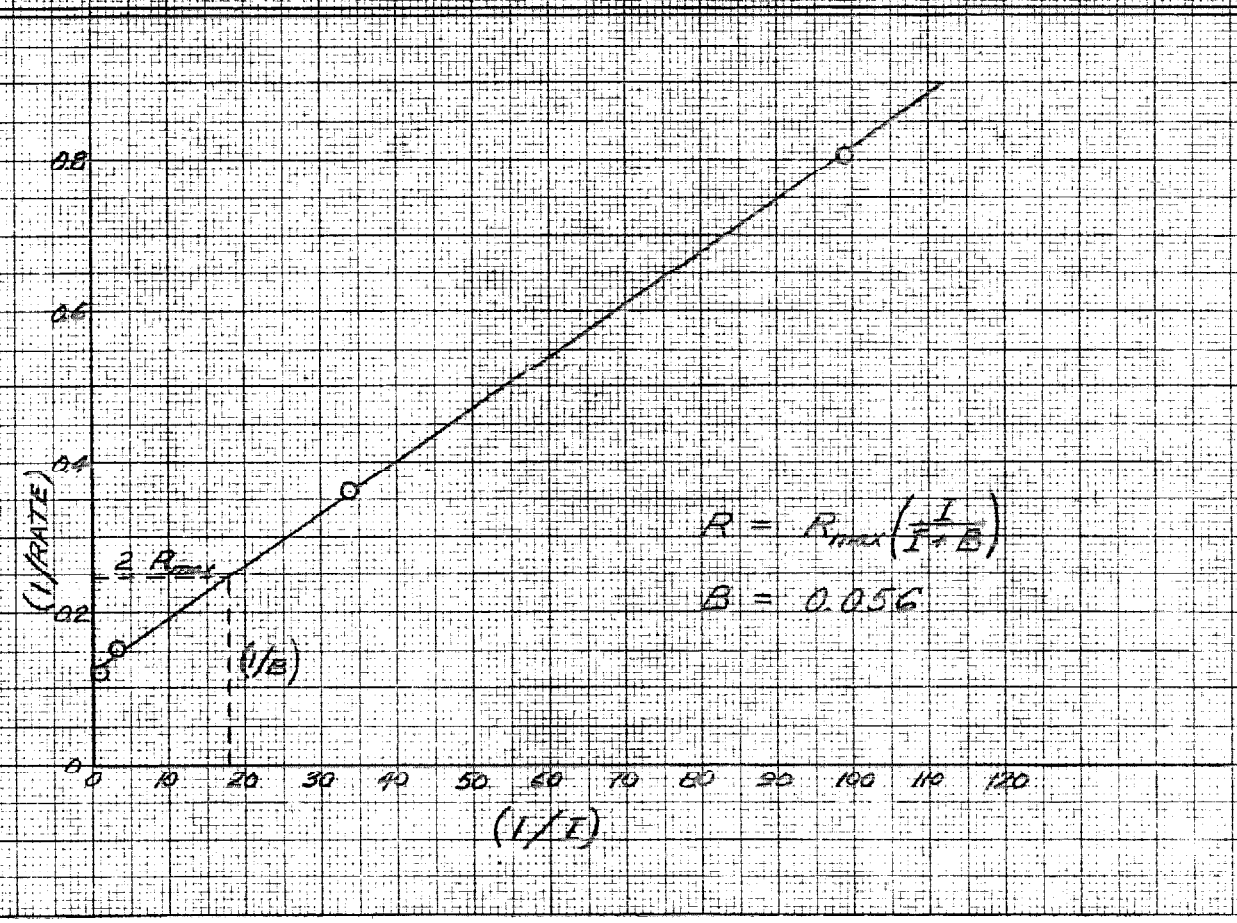
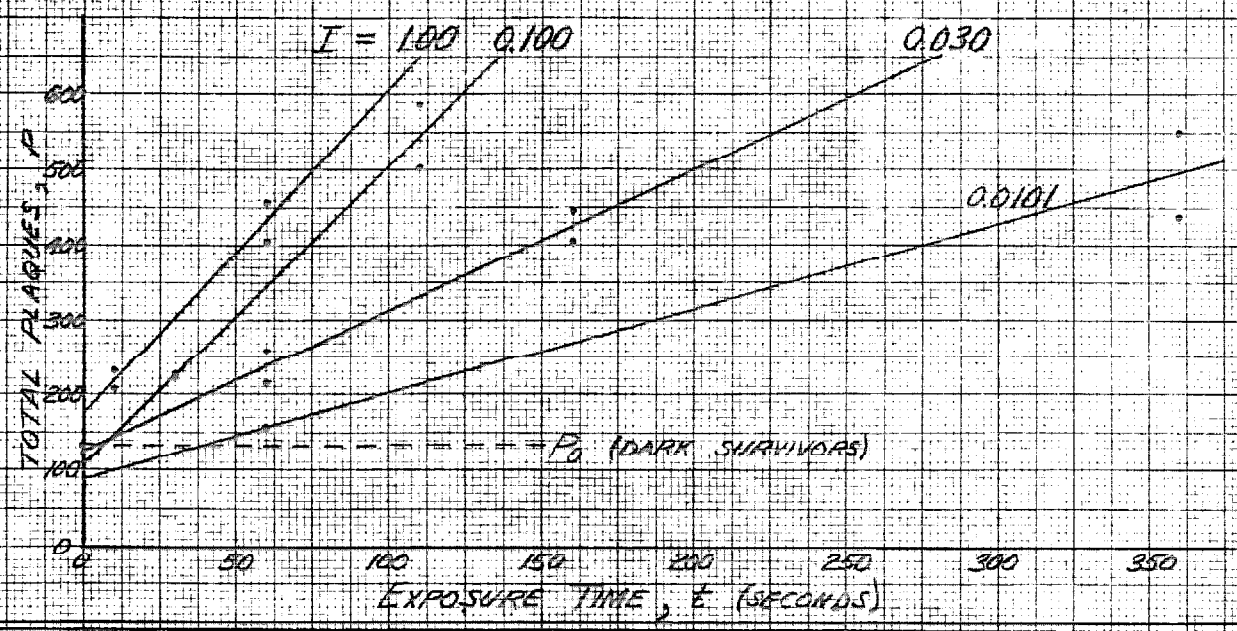


FIGURE 10: PHR RATE V.S. LIGHT INTENSITY; $T = 37.6^{\circ}C$

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I.



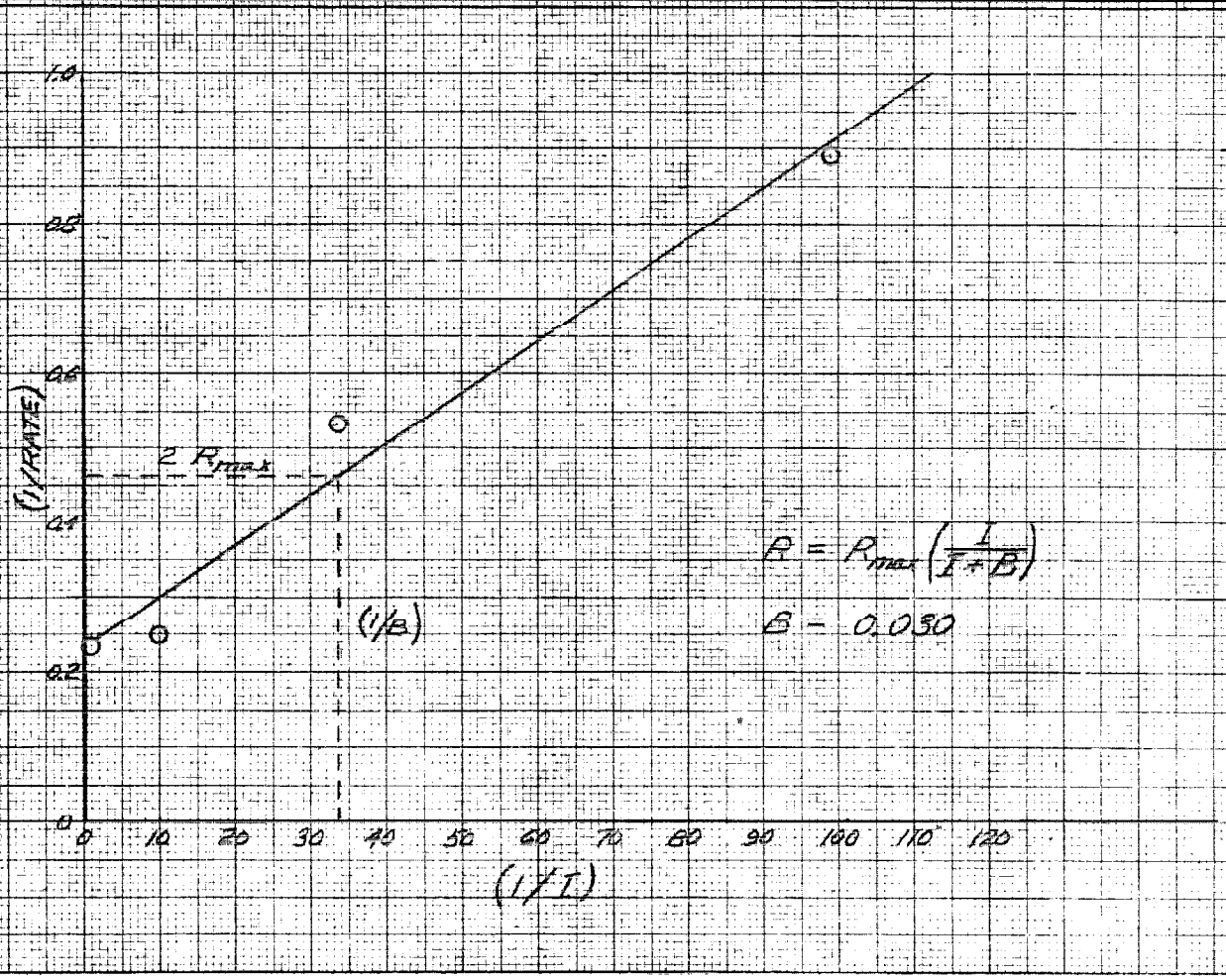
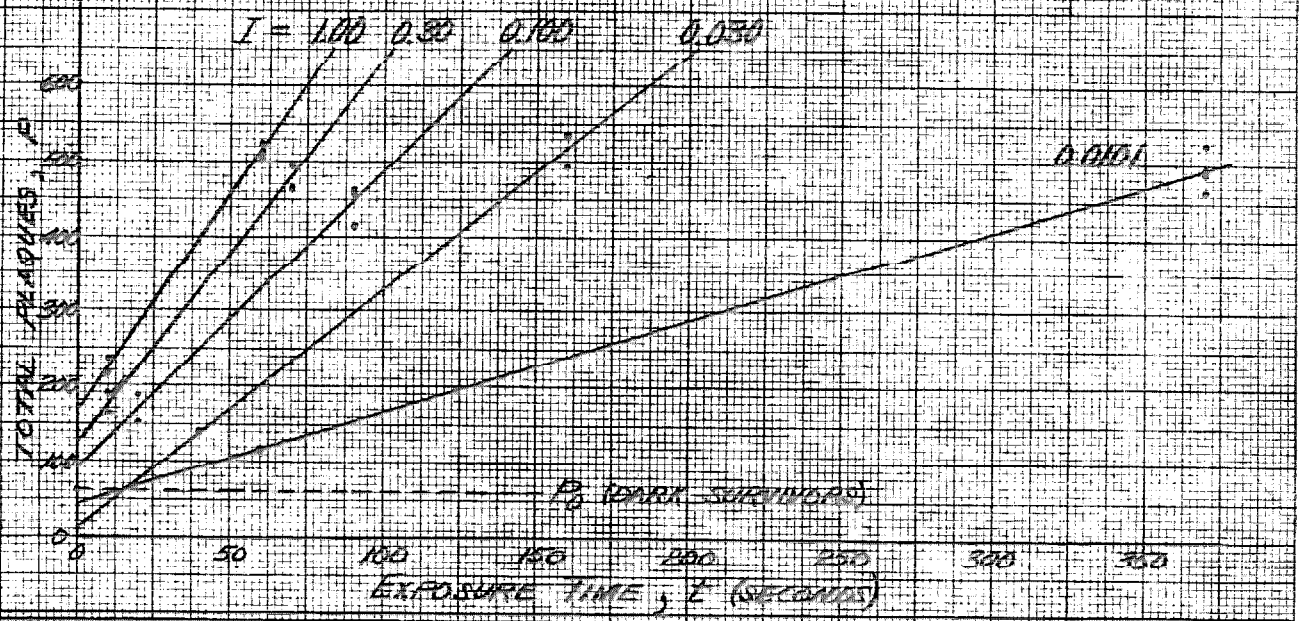


FIGURE 11: PMR RATE VS. LIGHT INTENSITY; $\bar{T} = 37.0°C$

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I



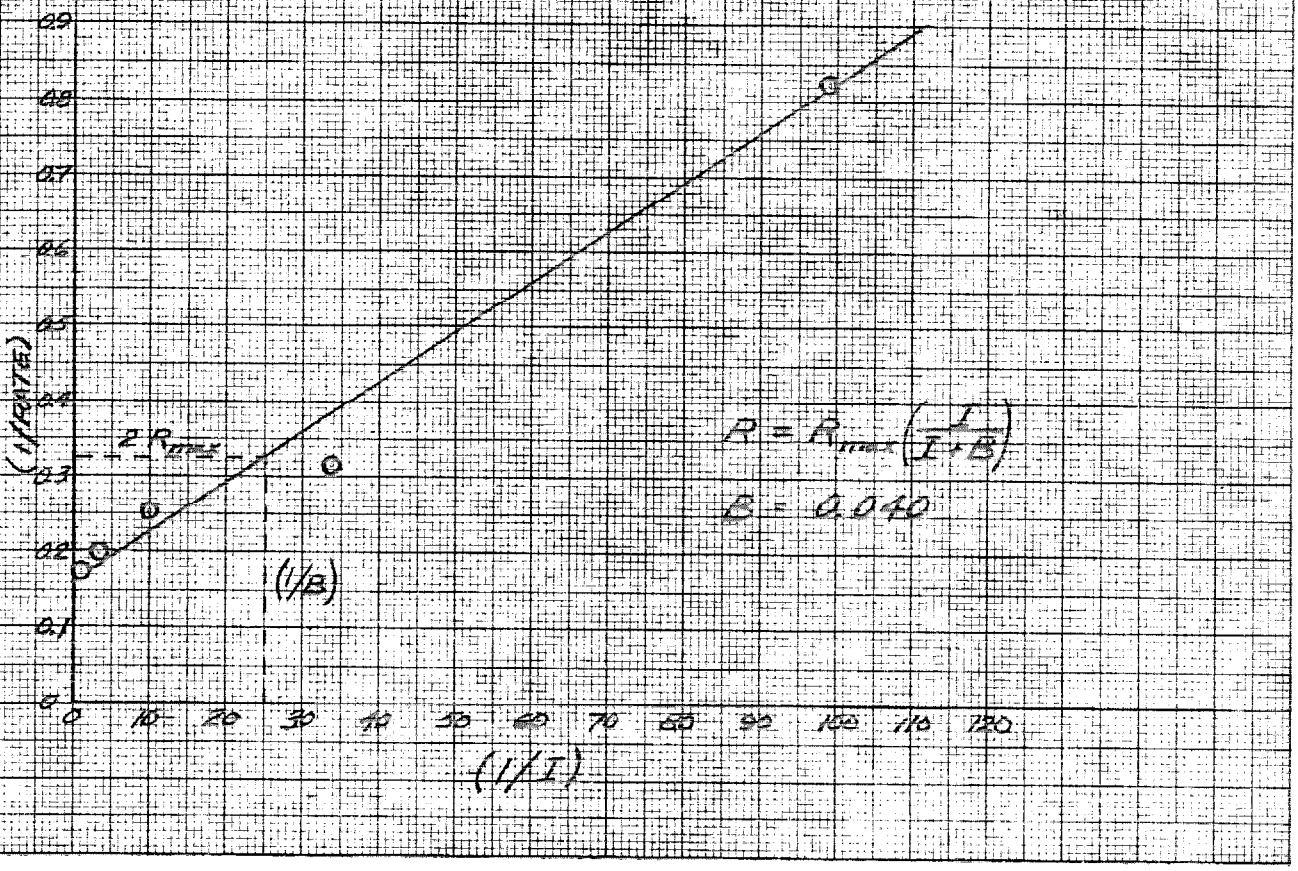
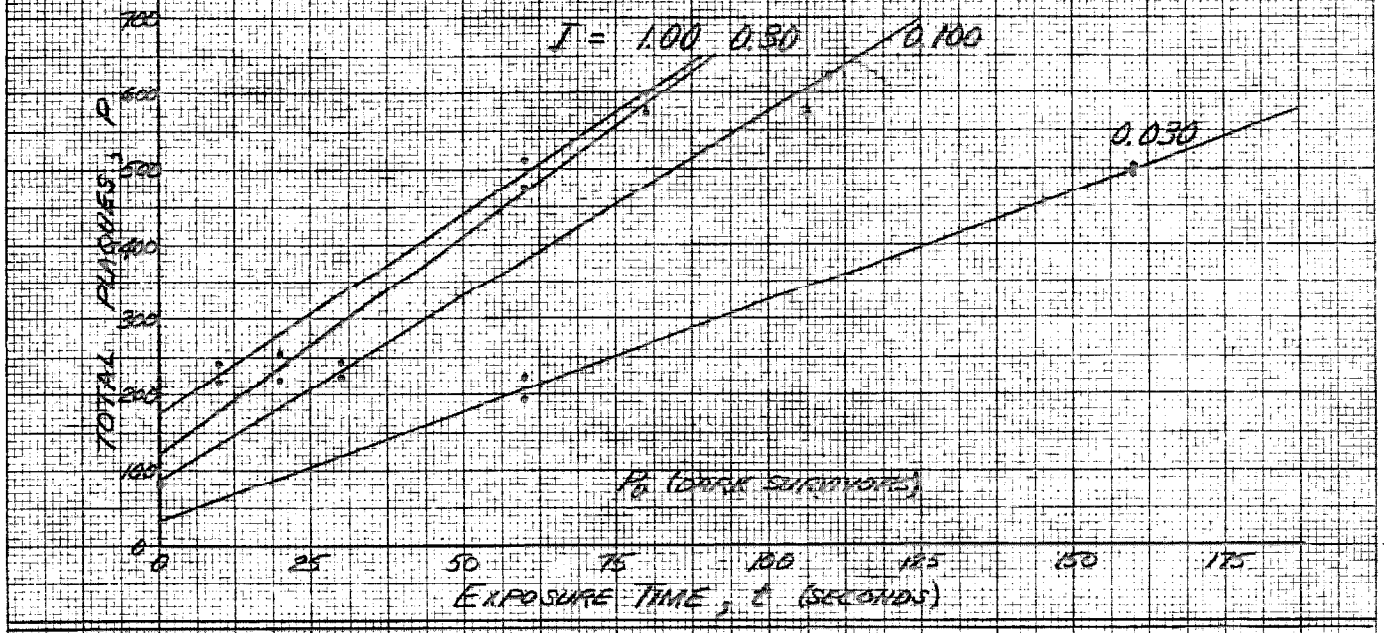


FIGURE 12: PHR RATE VS LIGHT INTENSITY; T = 36.6°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I.



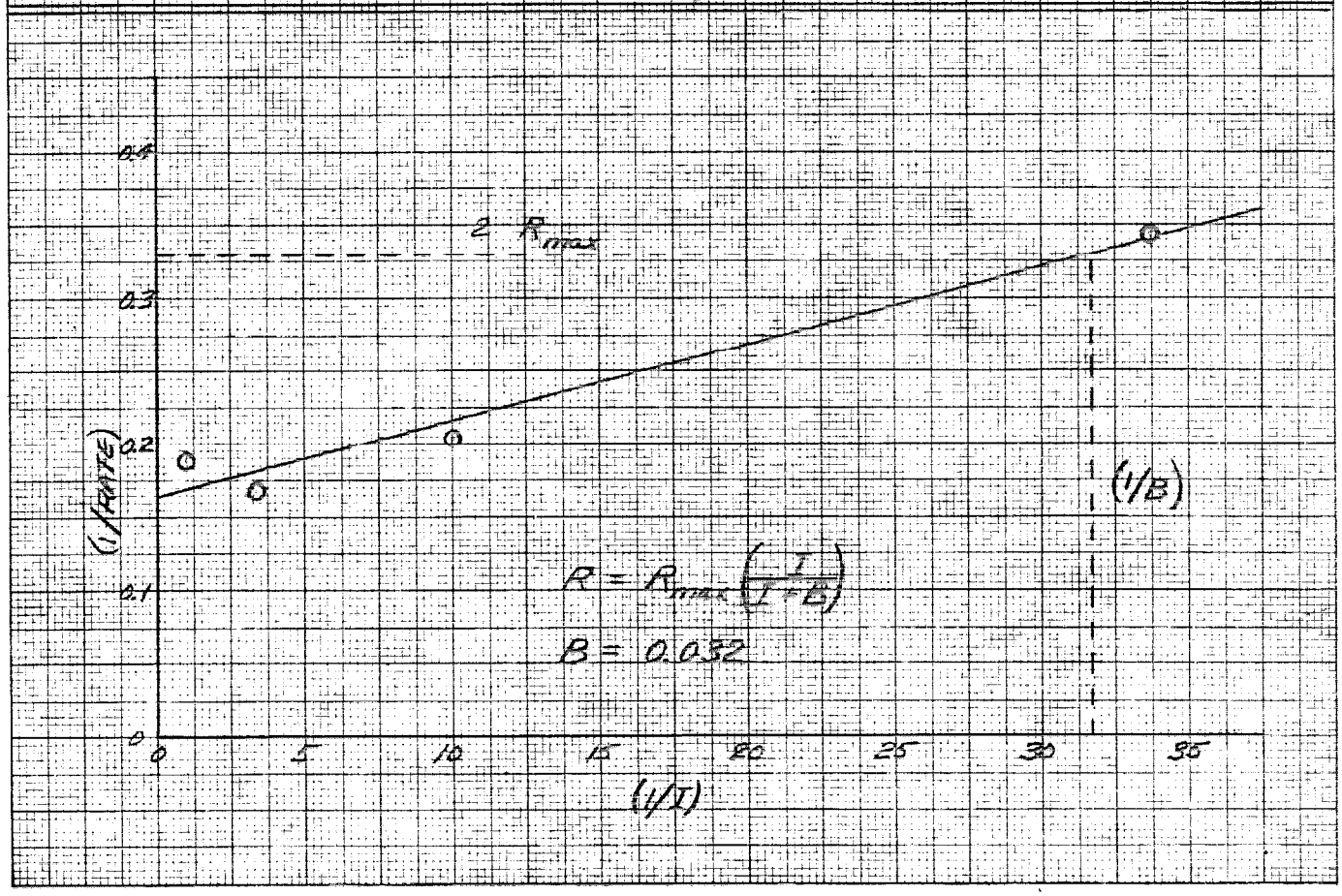
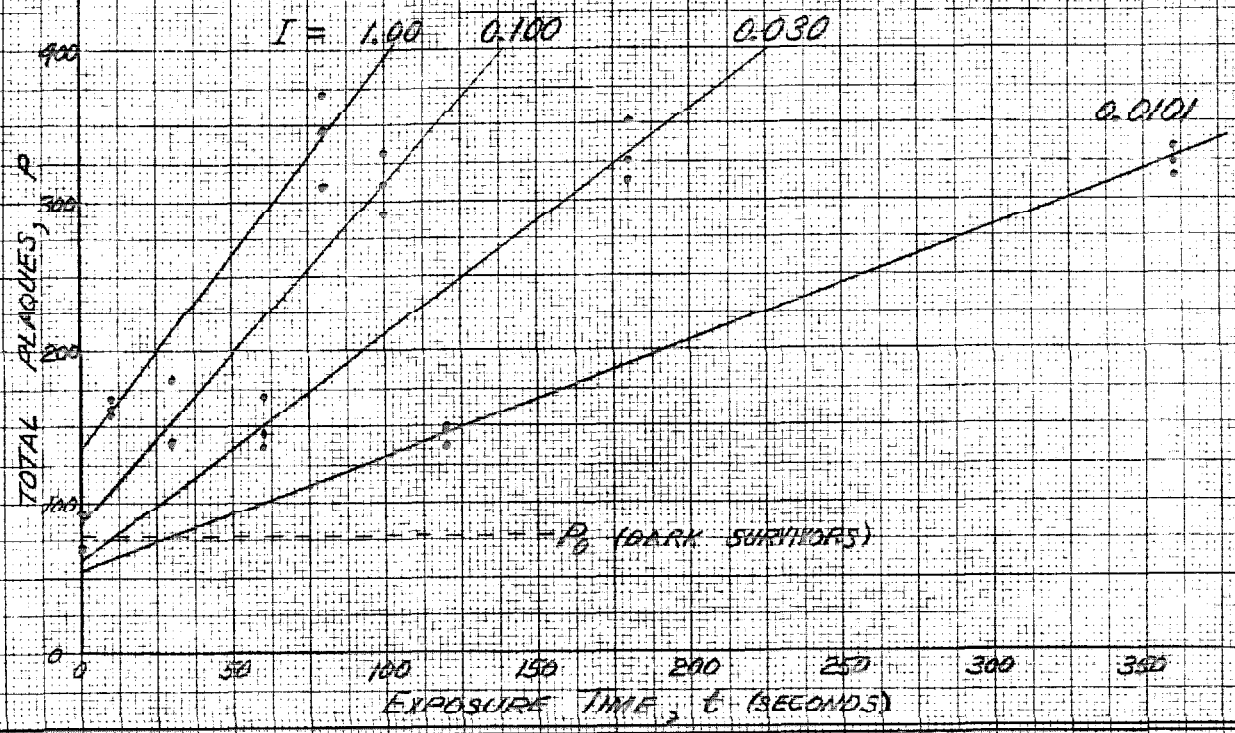


FIGURE 13: PHR RATE VS. LIGHT INTENSITY; $T = 30.4\,^{\circ}C$

SINGLE FLASH OF LENGTH t, INTENSITY I.



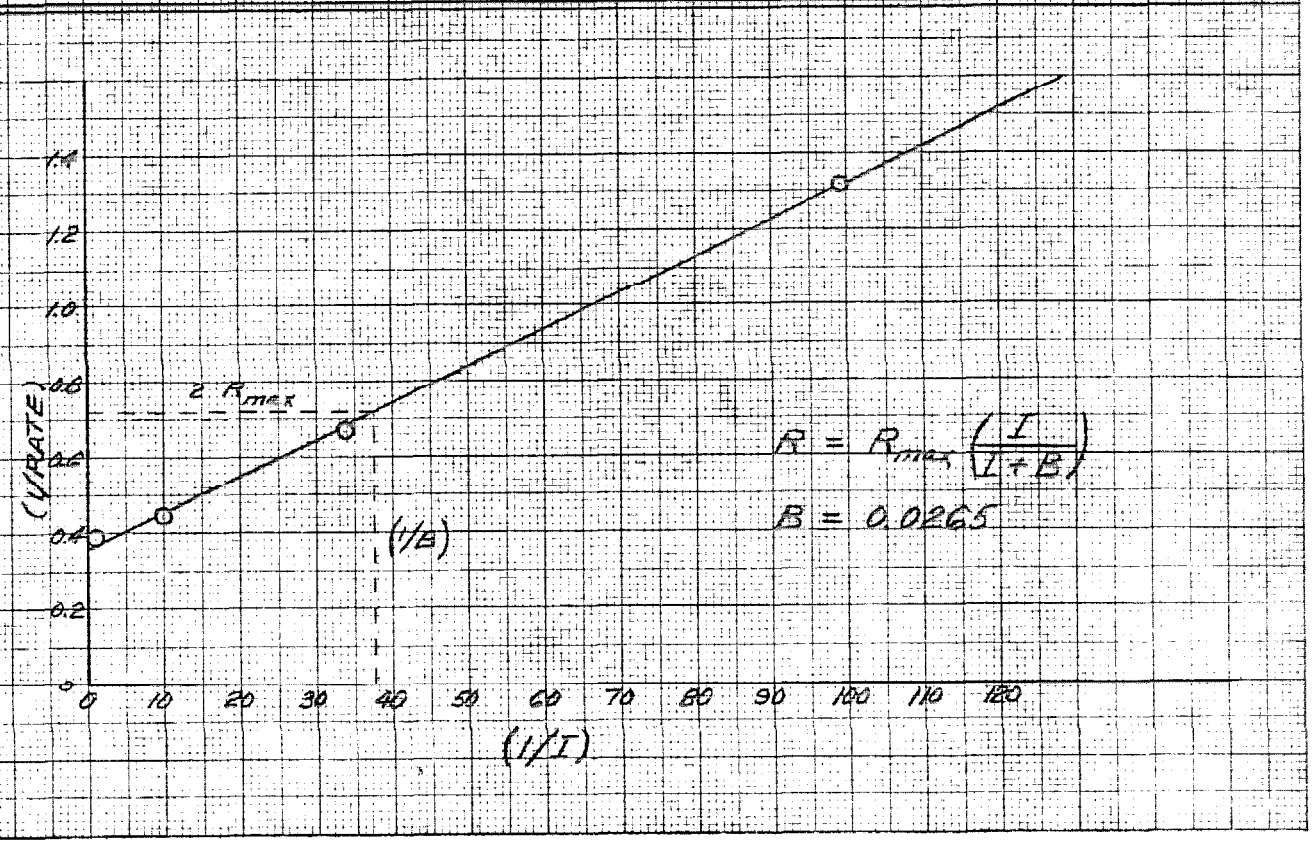
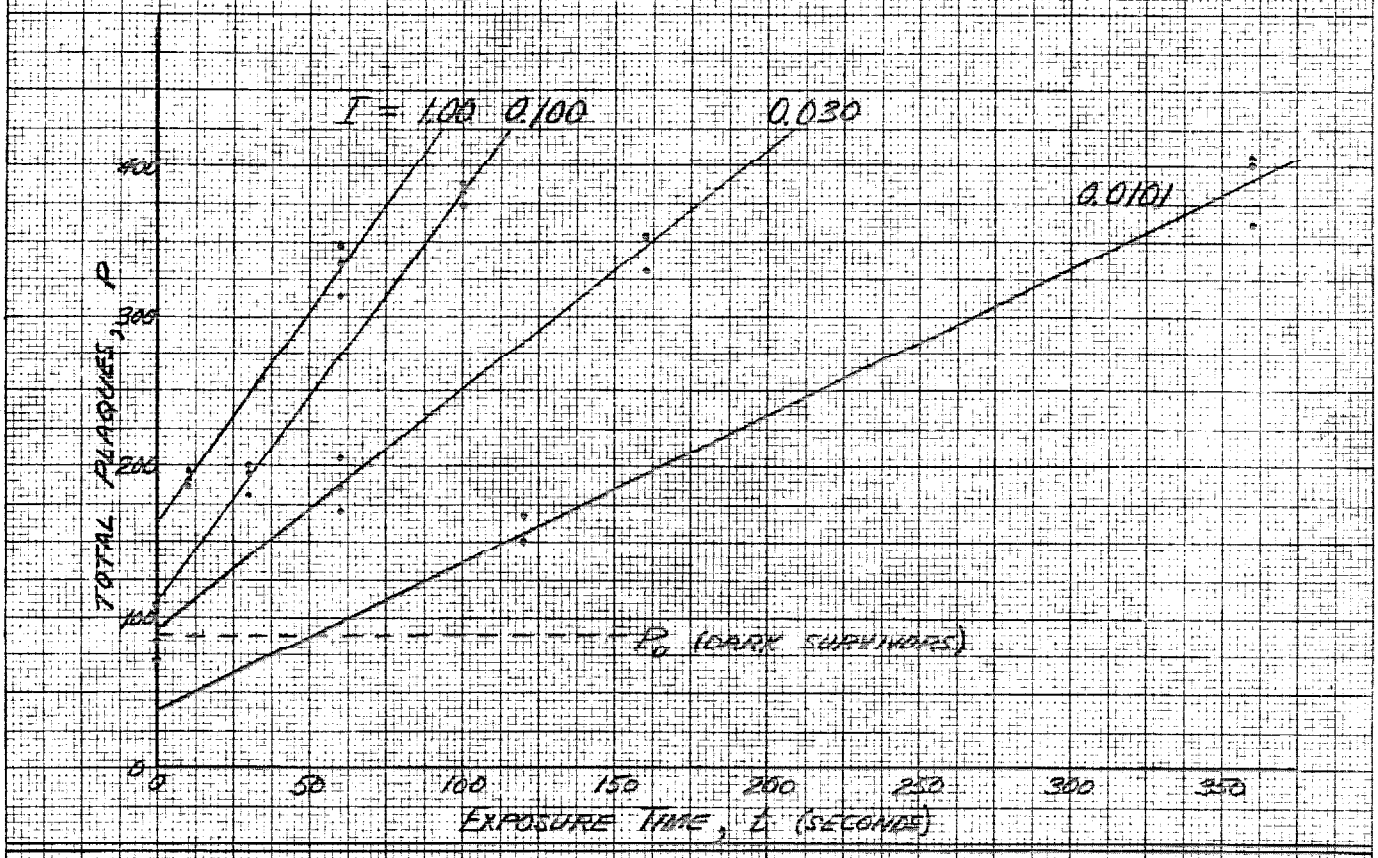


FIGURE 14: PMR RATE VS. LIGHT INTENSITY; T = 30.1 °C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I.



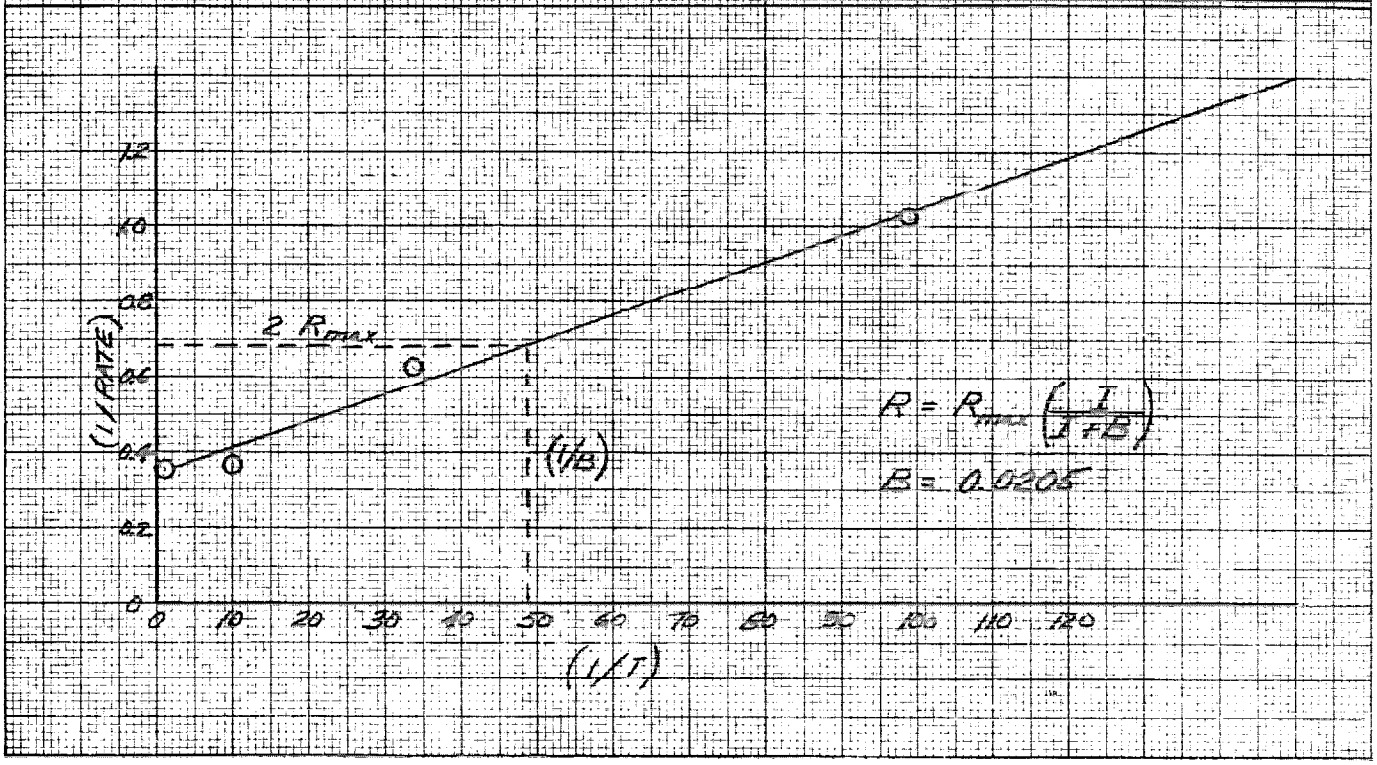
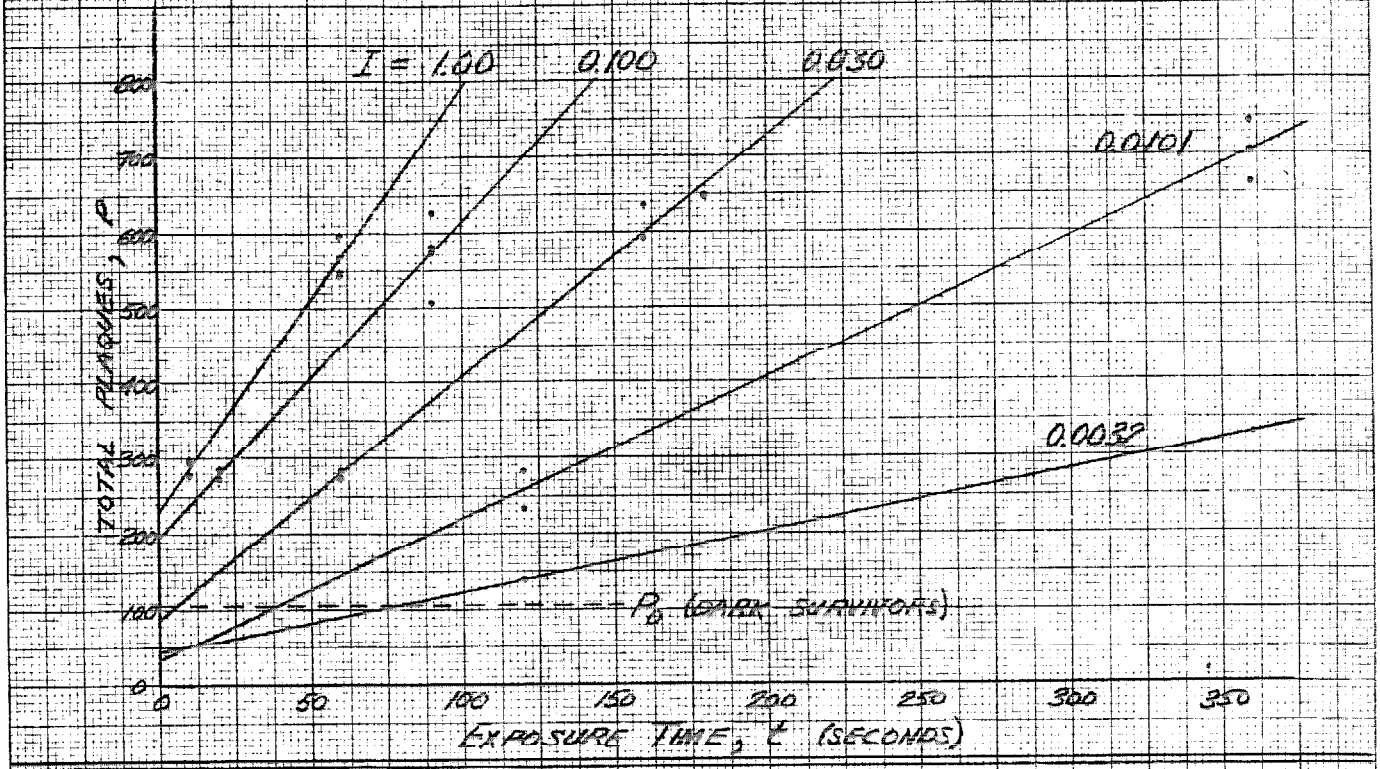


FIGURE 15: PHR RATE VS. LIGHT INTENSITY; $T = 300°C$

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I.



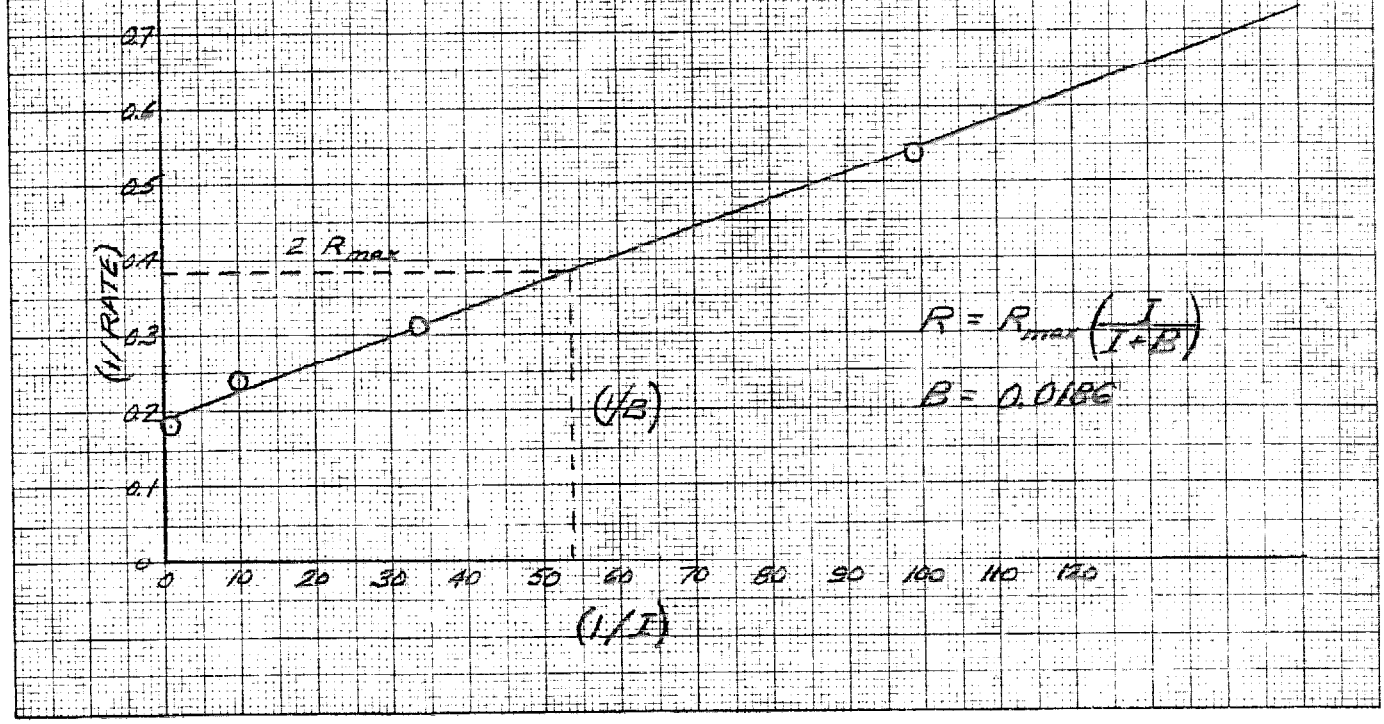
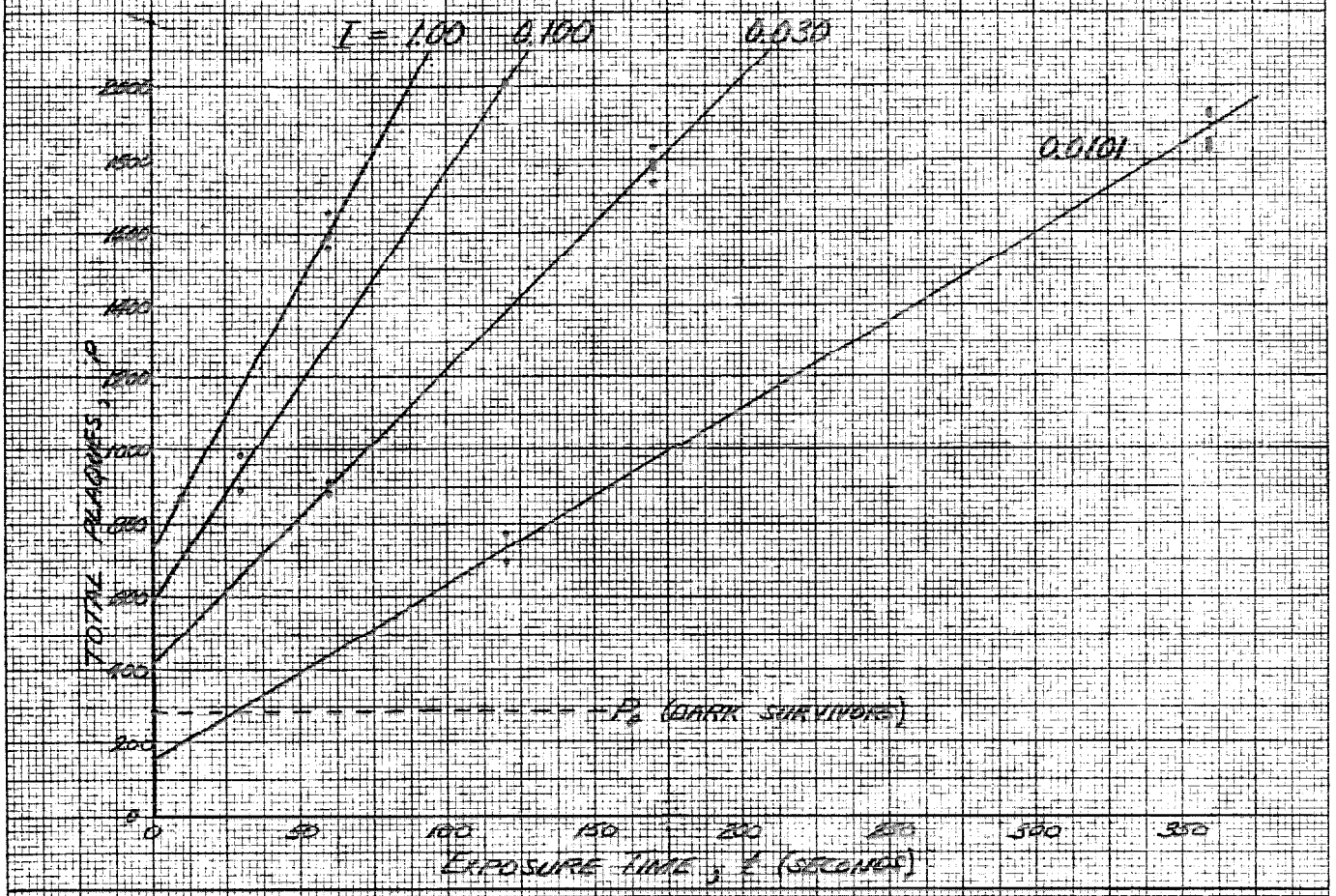


FIGURE K: PHR RATE VS. LIGHT INTENSITY; T = 29.9°C

SINGLE LIGHT FLASH OF LENGTH t_2, INTENSITY I.



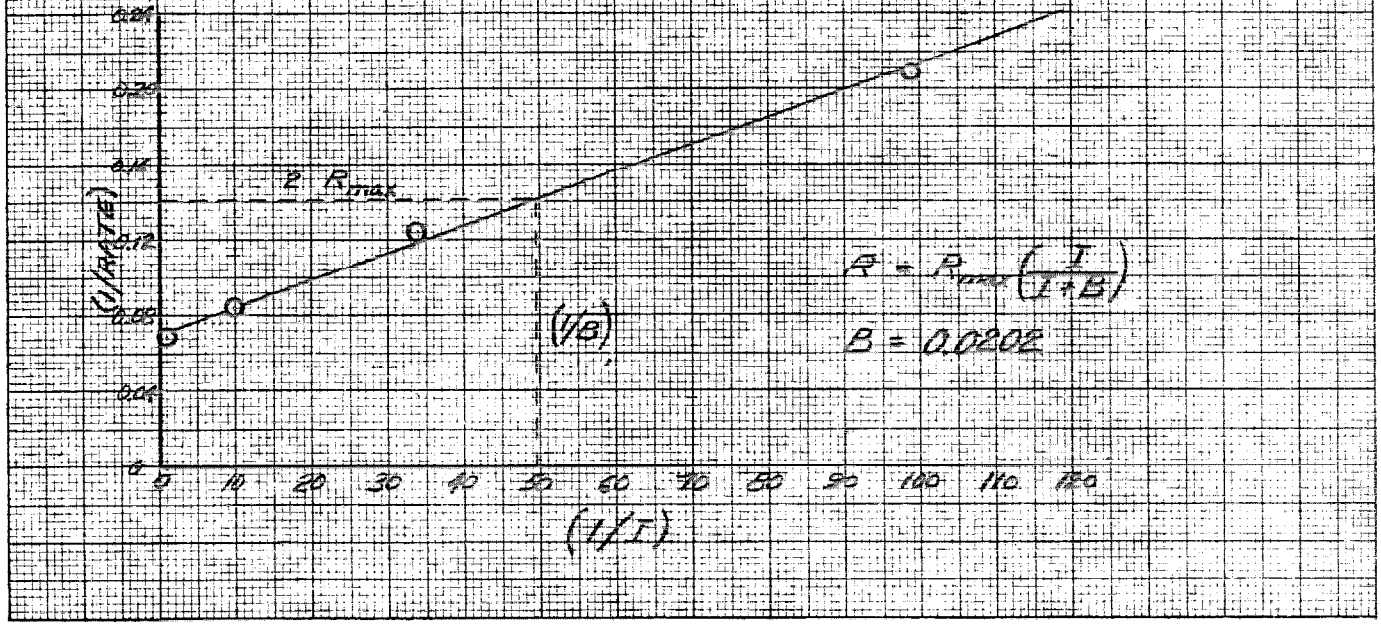
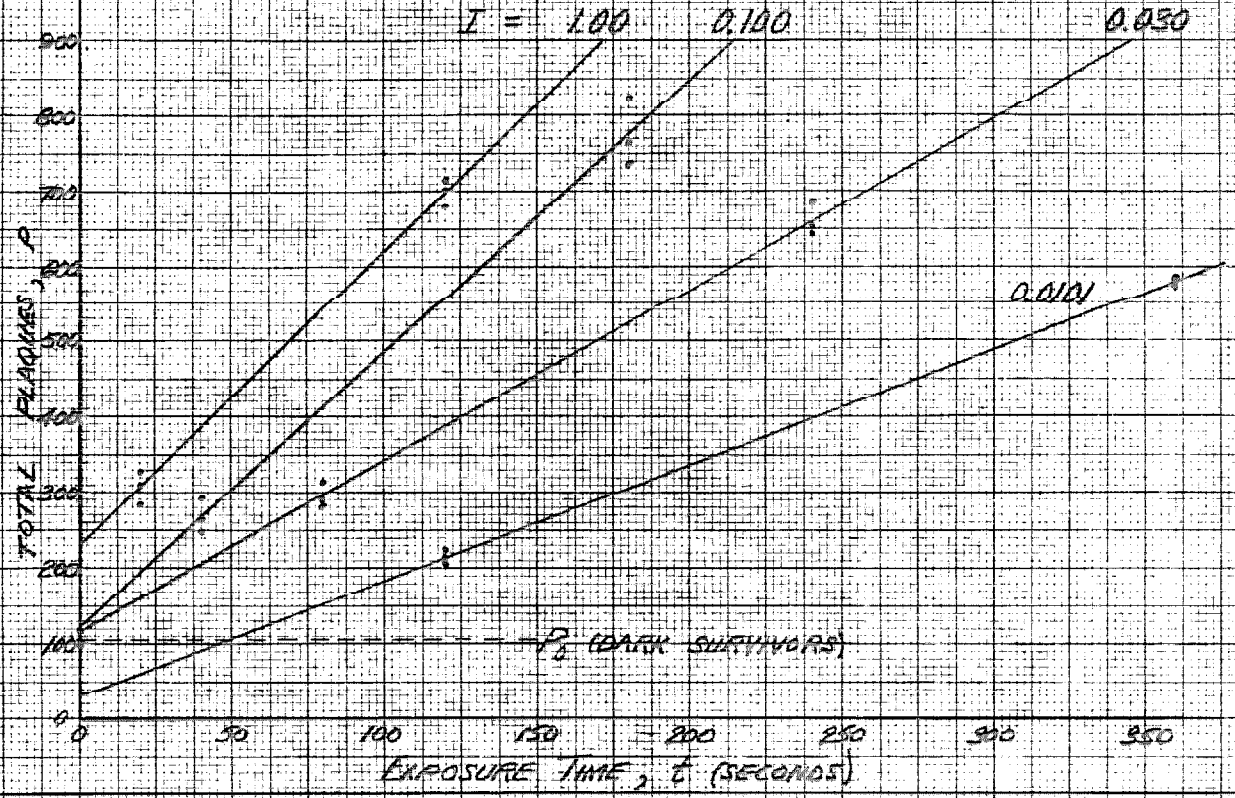


FIGURE 17. PMR RATE VS. LIGHT INTENSITY; $T = 23.2°C$

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I.



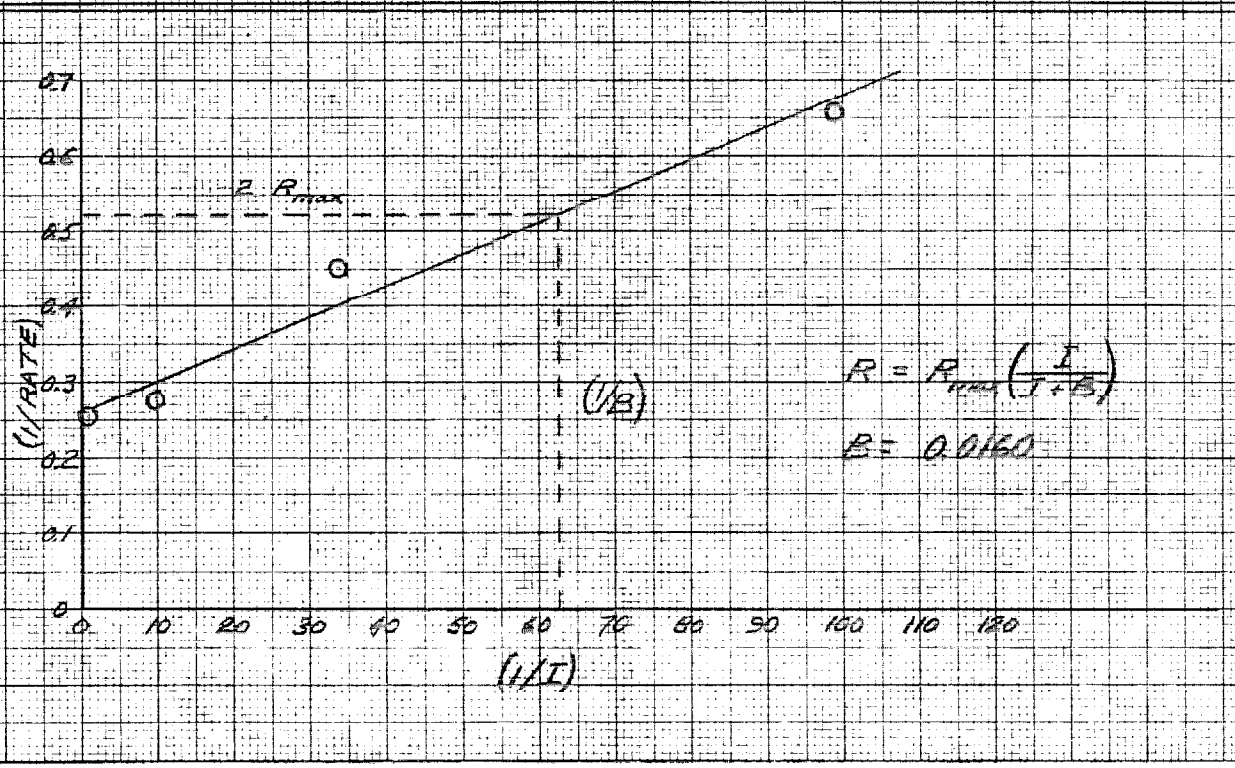
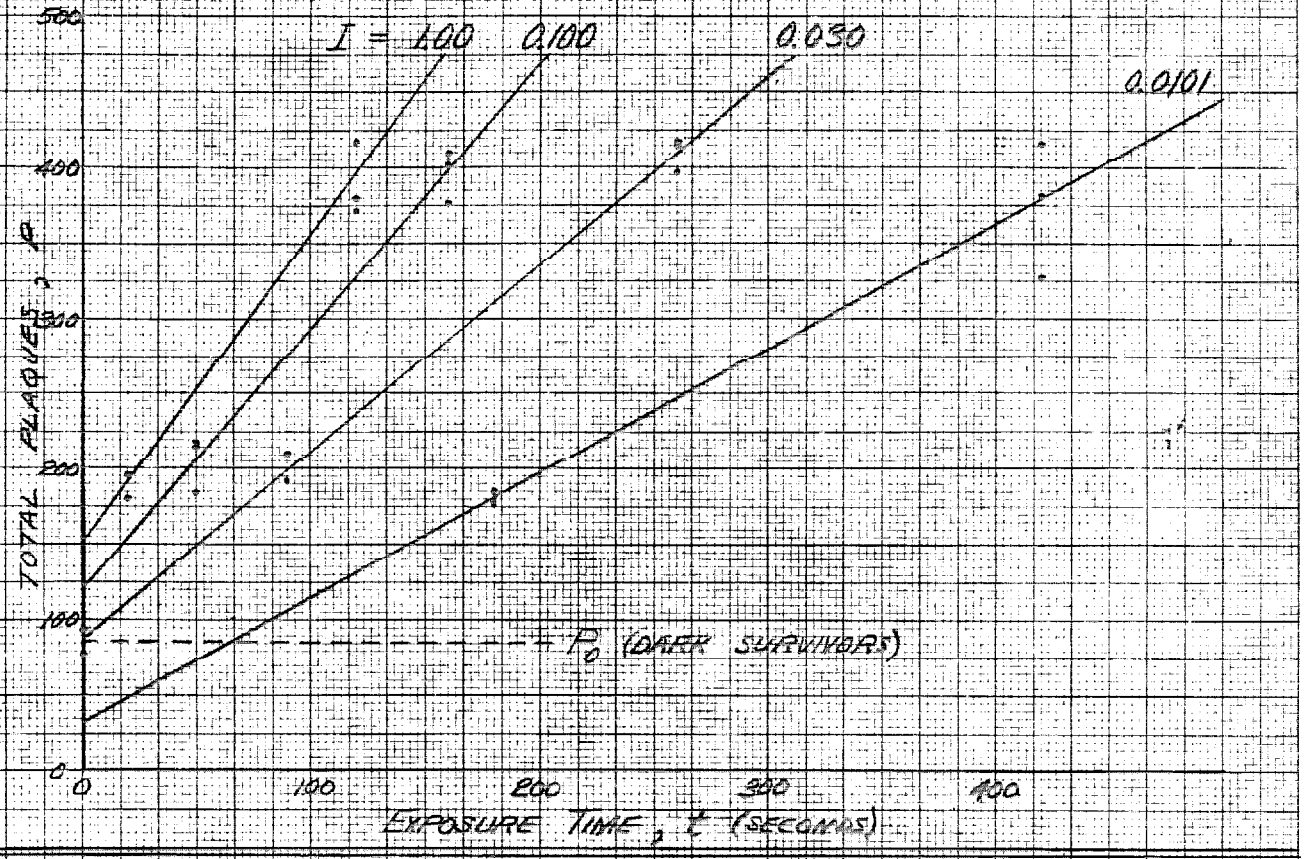


FIGURE 18: PHR RATE VS LIGHT INTENSITY; T = 22.8°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I.



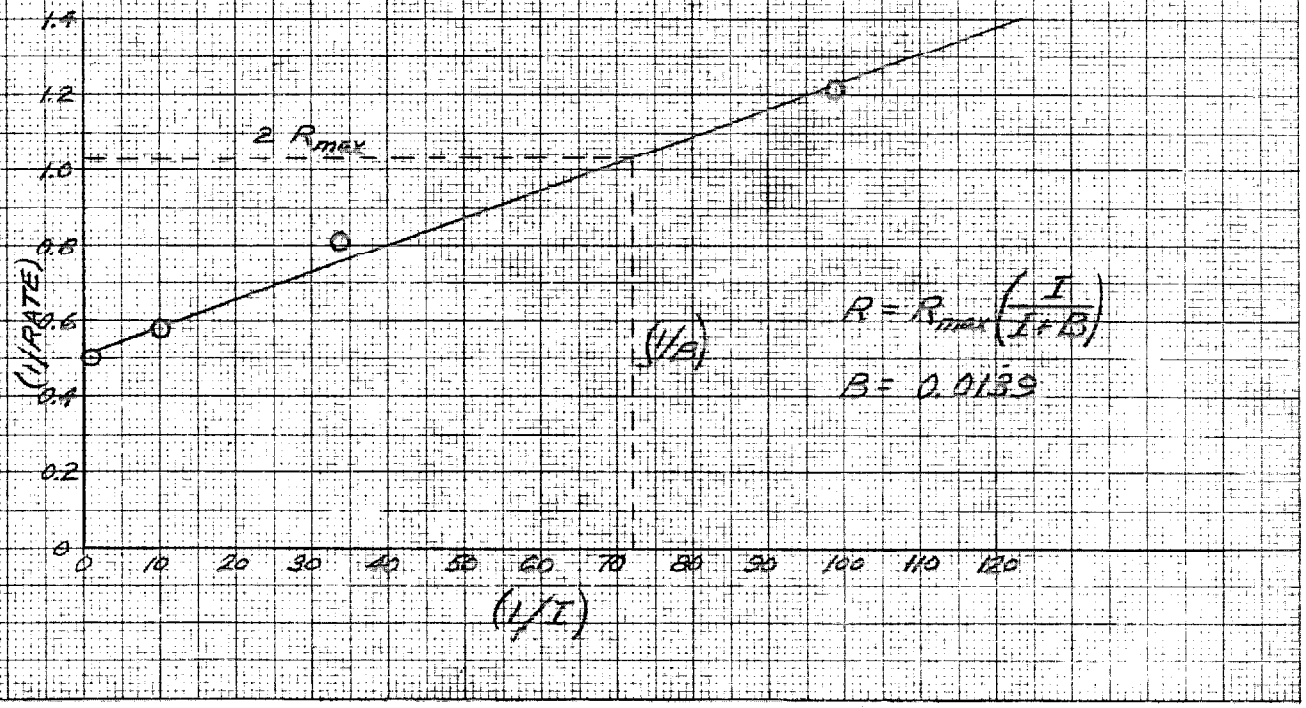
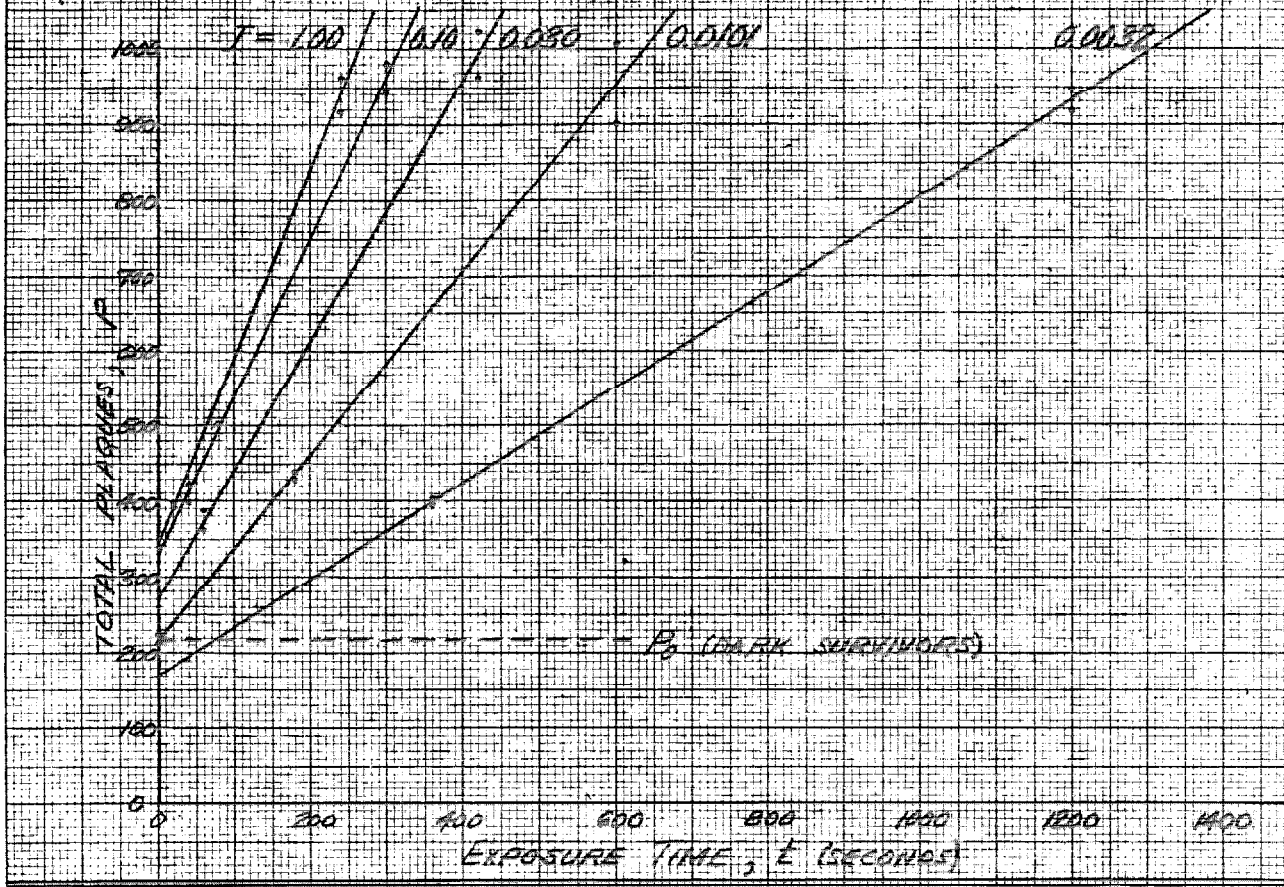


FIGURE 10: EMR RATE VS. LIGHT INTENSITY; T = 14.7°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I.



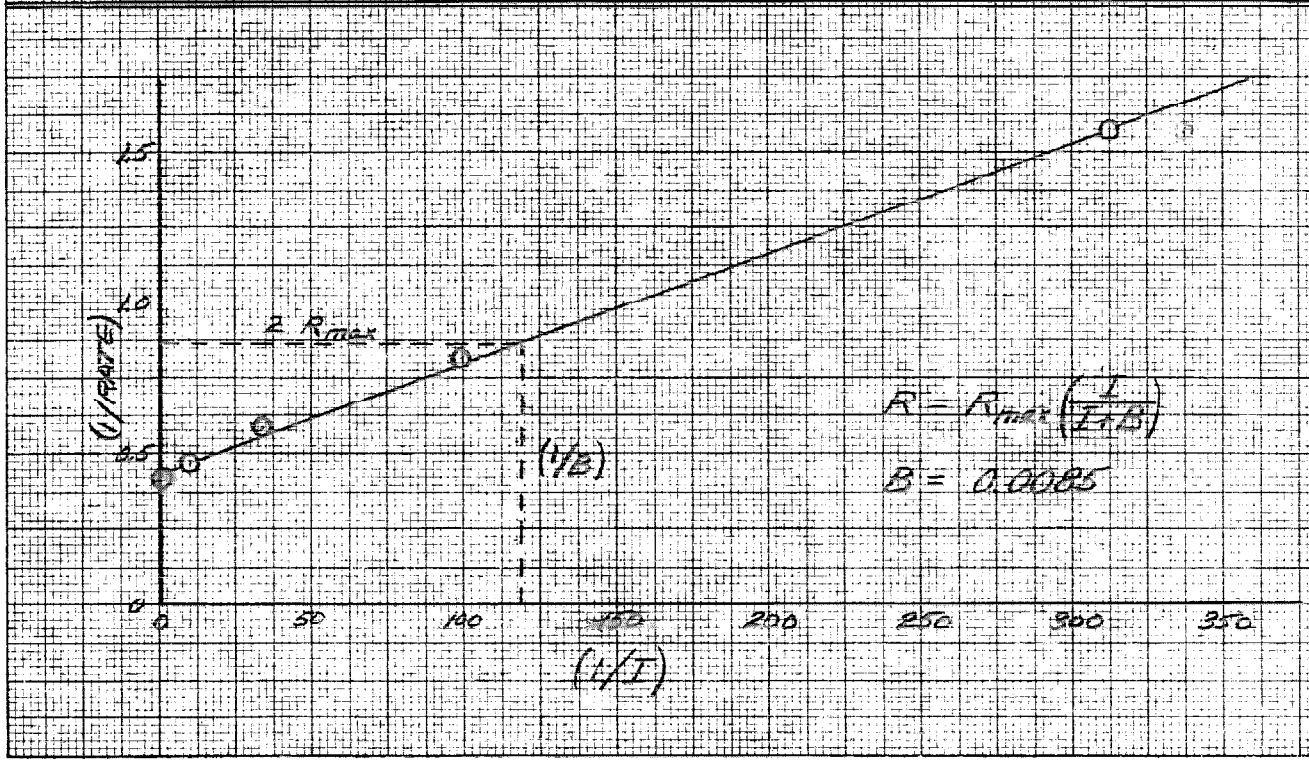
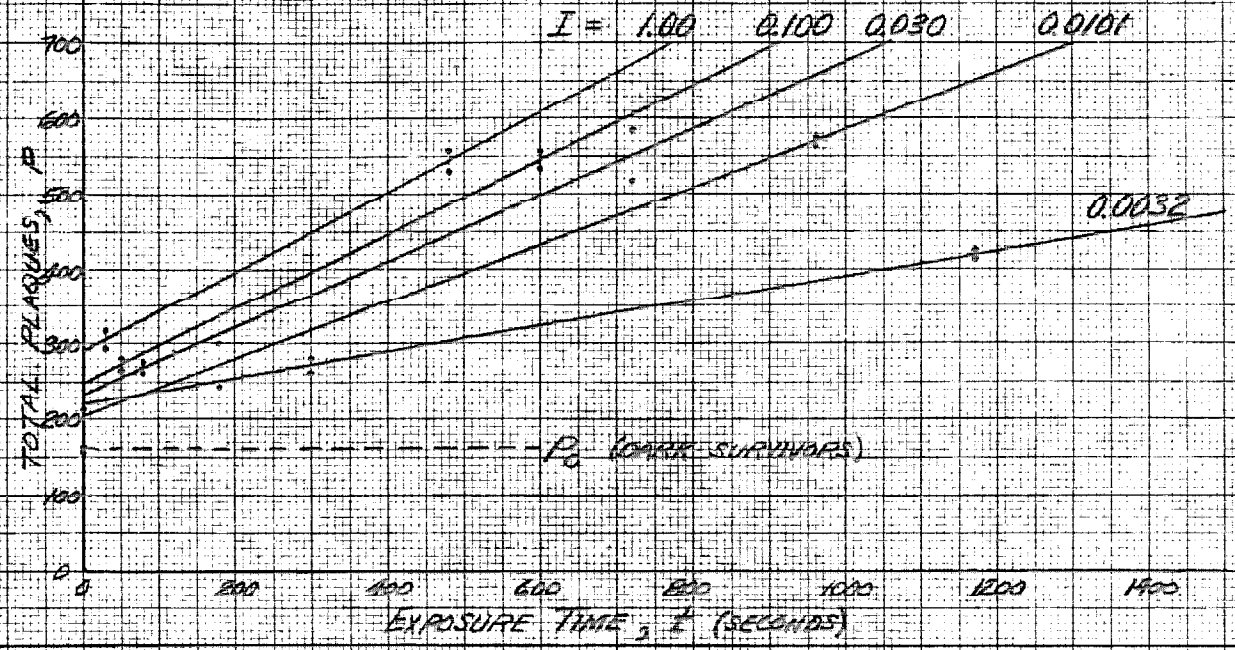


FIGURE 20. PHR RATE VS. LIGHT INTENSITY; $T = 8.2\,^{\circ}C$

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY I



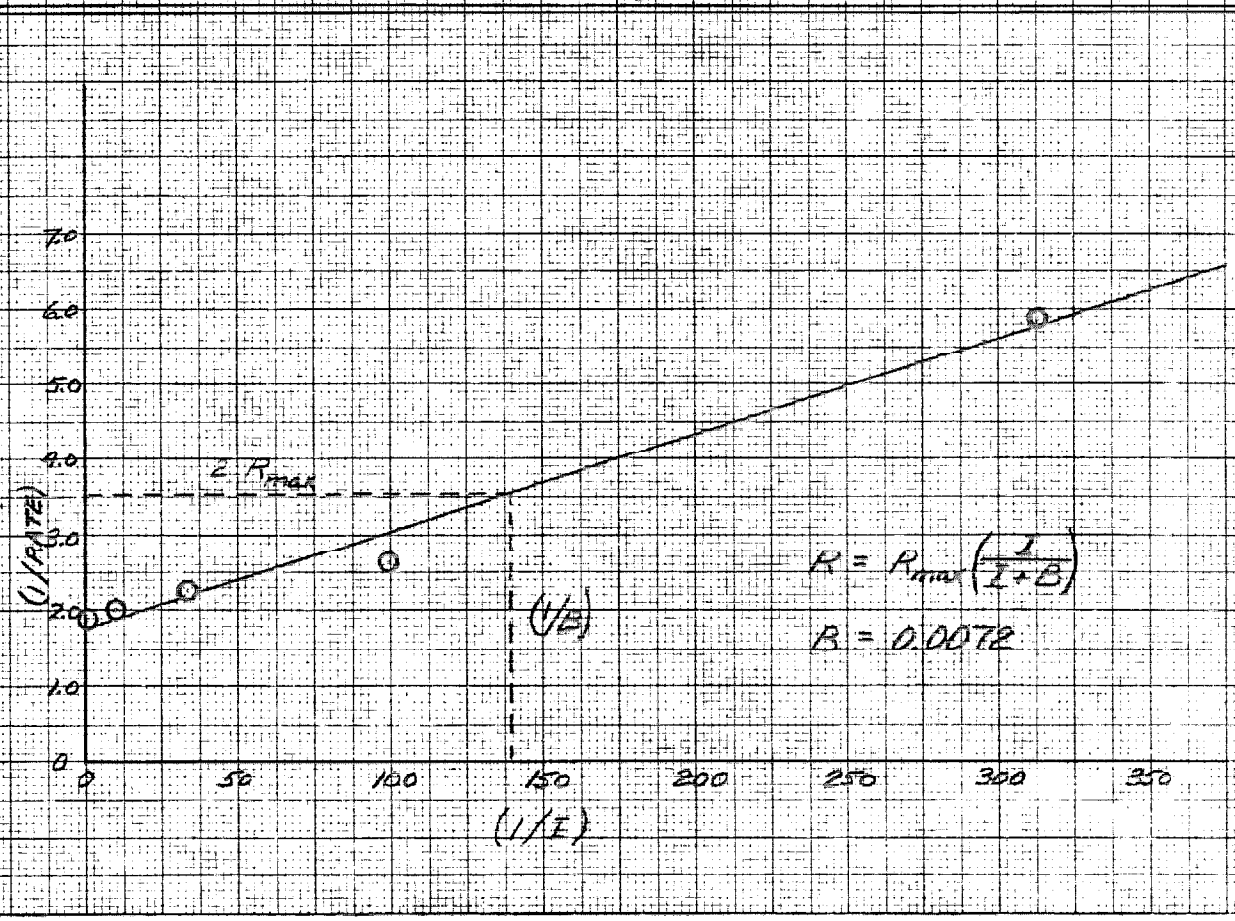
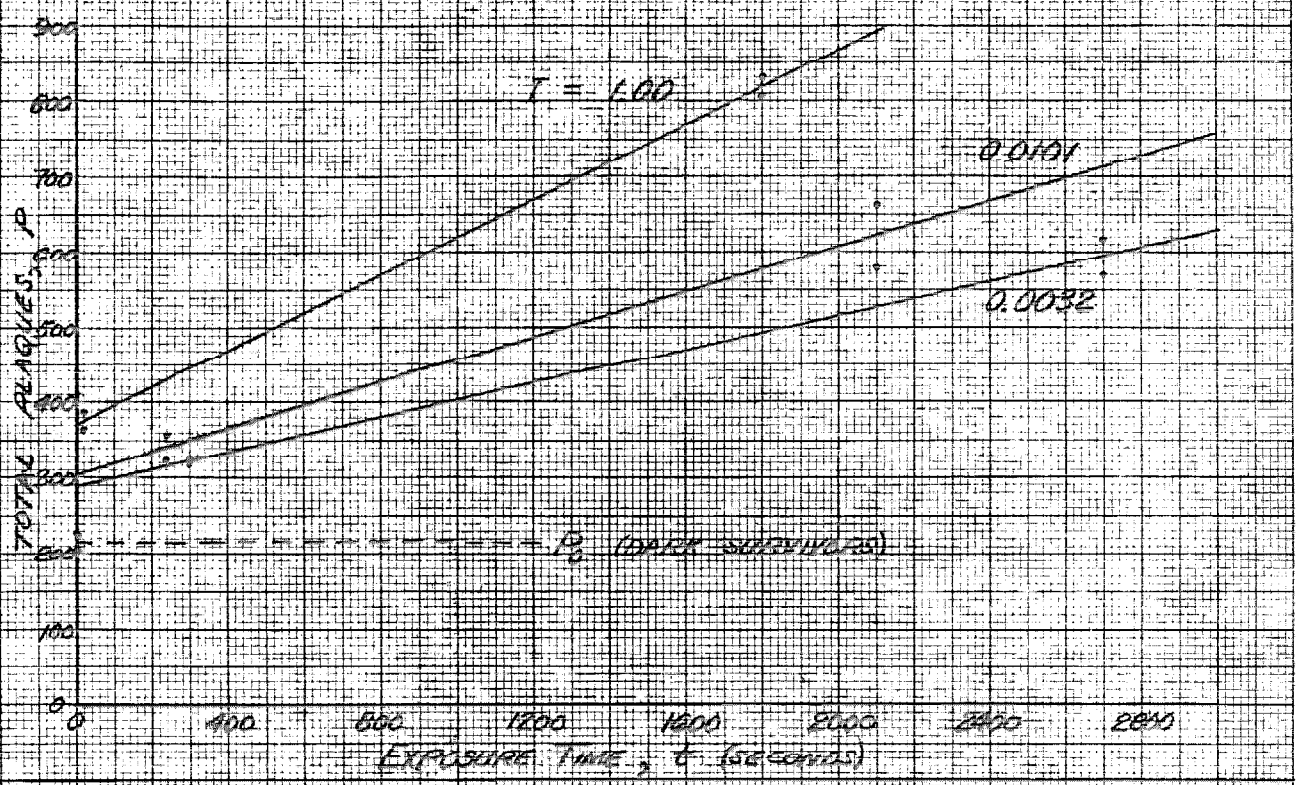


FIGURE 21: PHR RATE VS. LIGHT INTENSITY; $T = 0.0°C$

SINGLE LIGHT FLASH OF LENGTH t_1, INTENSITY I.



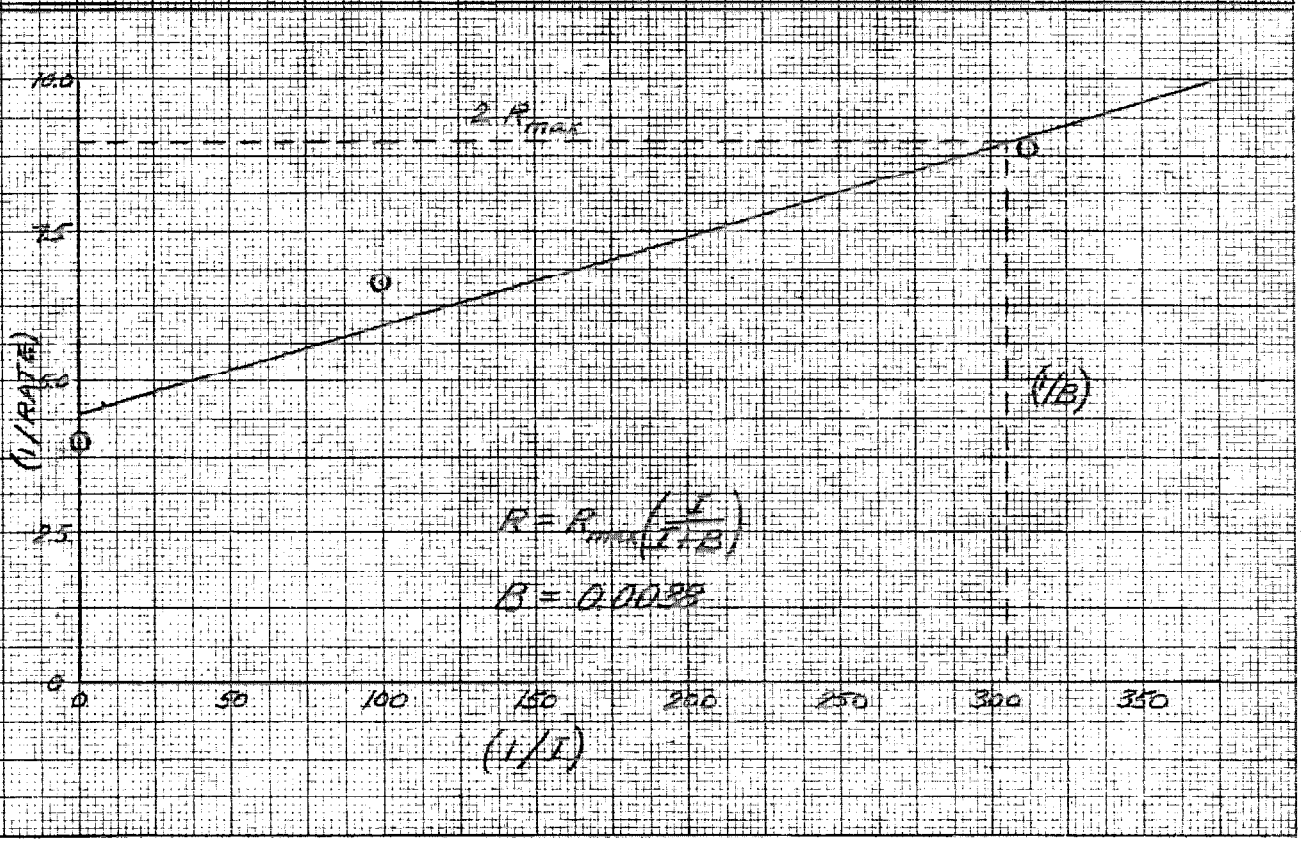


FIGURE 22: MAXIMUM PHR RATE AT 45.7°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY $I = 100$

$$\frac{R(10, 45.7^\circ)}{R(10, 36.6^\circ)} = 1.68$$

$$\frac{R_{max}(45.7^\circ)}{R_{max}(36.6^\circ)} = \left(\frac{1.66}{1.03}\right)(1.68) = 1.72$$

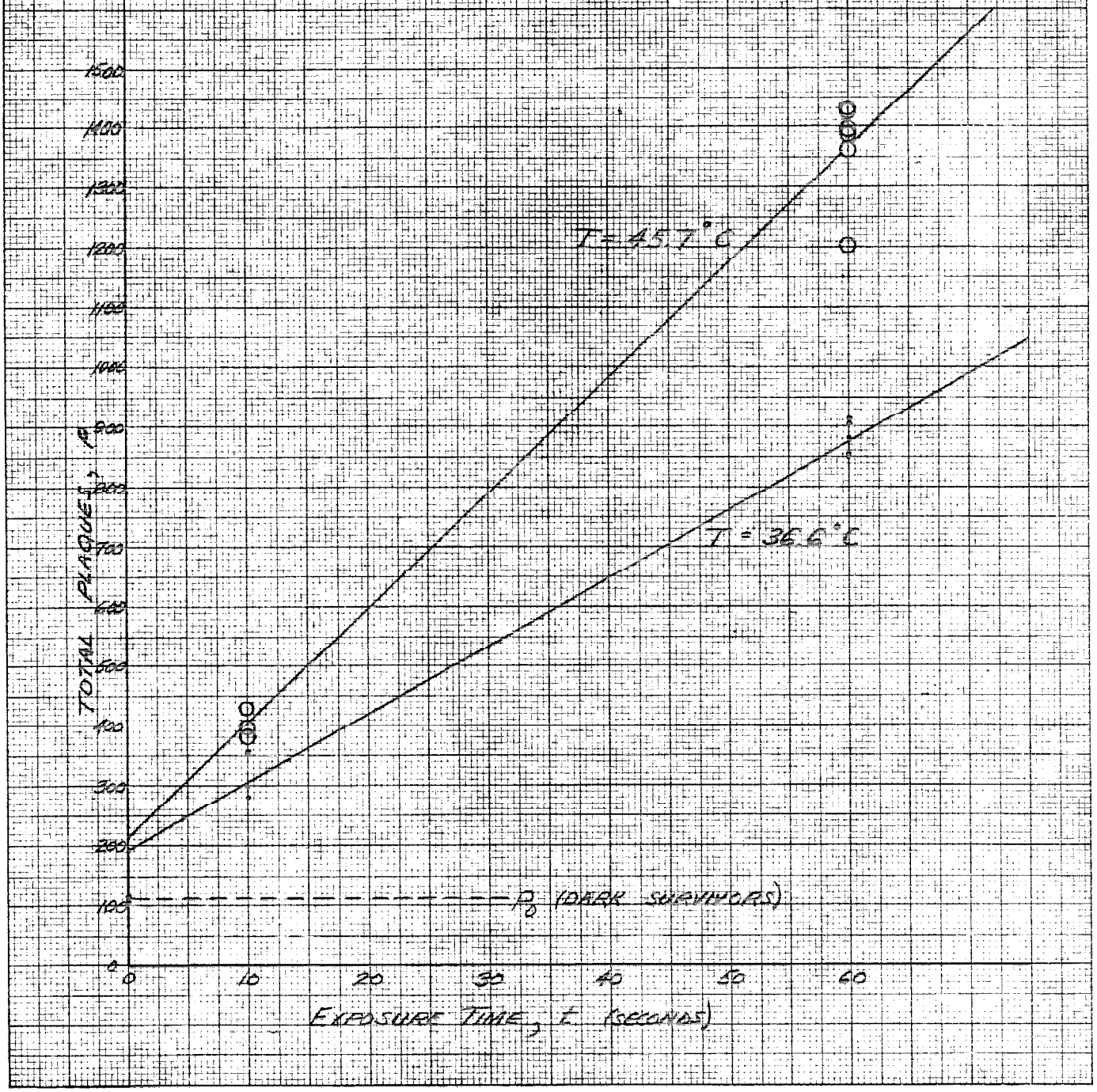


FIGURE 23. MAXIMUM PUR RATE AT 44.7°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY $I = 100$

$$\frac{R(10, 44.7^\circ)}{R(10, 37.8^\circ)} = 1.45$$

$$\therefore \frac{R_{max}(44.7^\circ)}{R_{max}(36.6^\circ)} = \left(\frac{1.06}{1.03}\right)(1.45) = 1.49$$

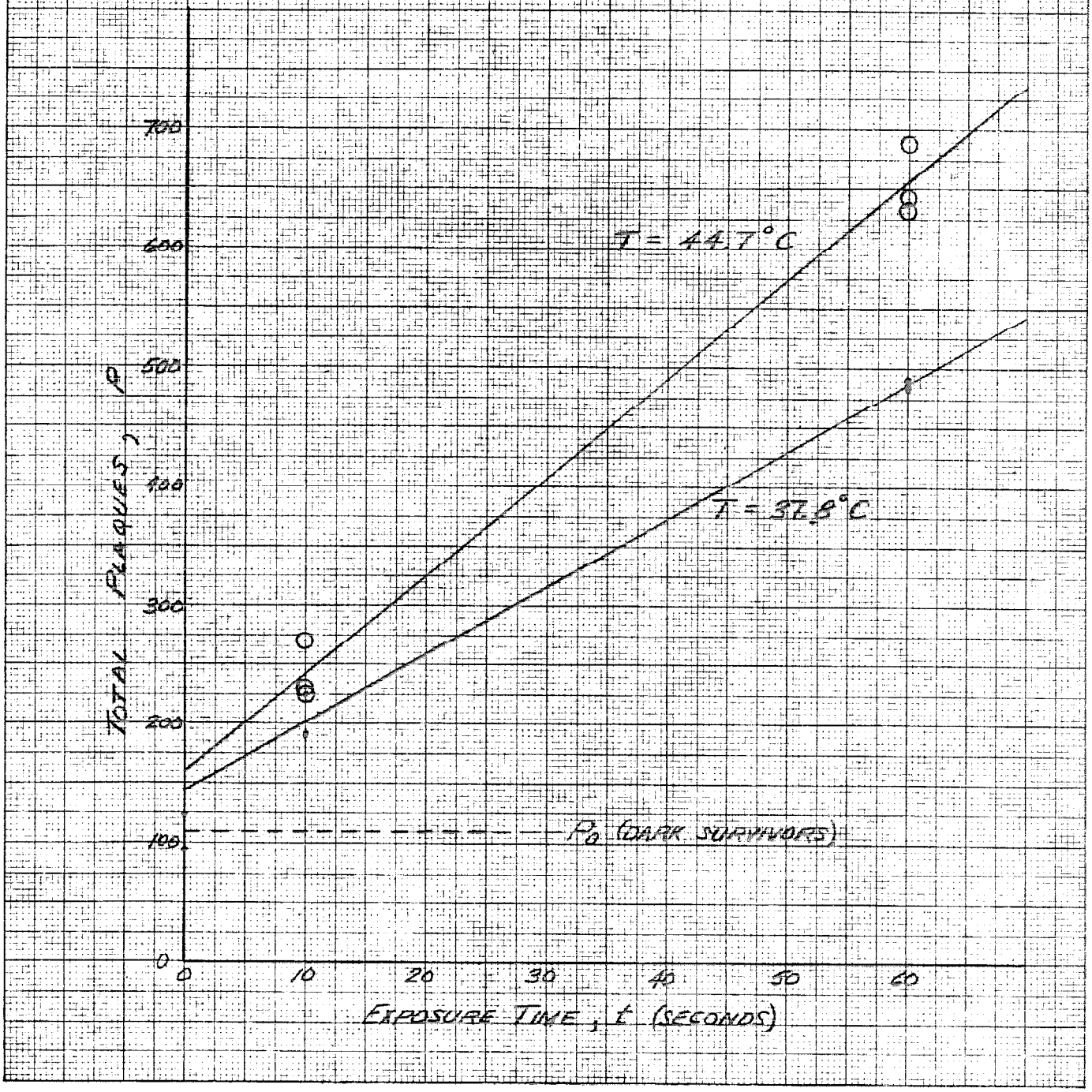


FIGURE 24: MAXIMUM PHR RATE AT 30.0°C

SINGLE LIGHT FLASH OF LENGTH t_1, INTENSITY $I = 100$

$$\frac{R(1.0, 30.0°)}{R(1.0, 34.6°)} = 0.65$$

$$\therefore \frac{R_{max}(30.0°)}{R_{max}(34.6°)} = \left(\frac{1.02}{1.03}\right)(0.63) = 0.62$$

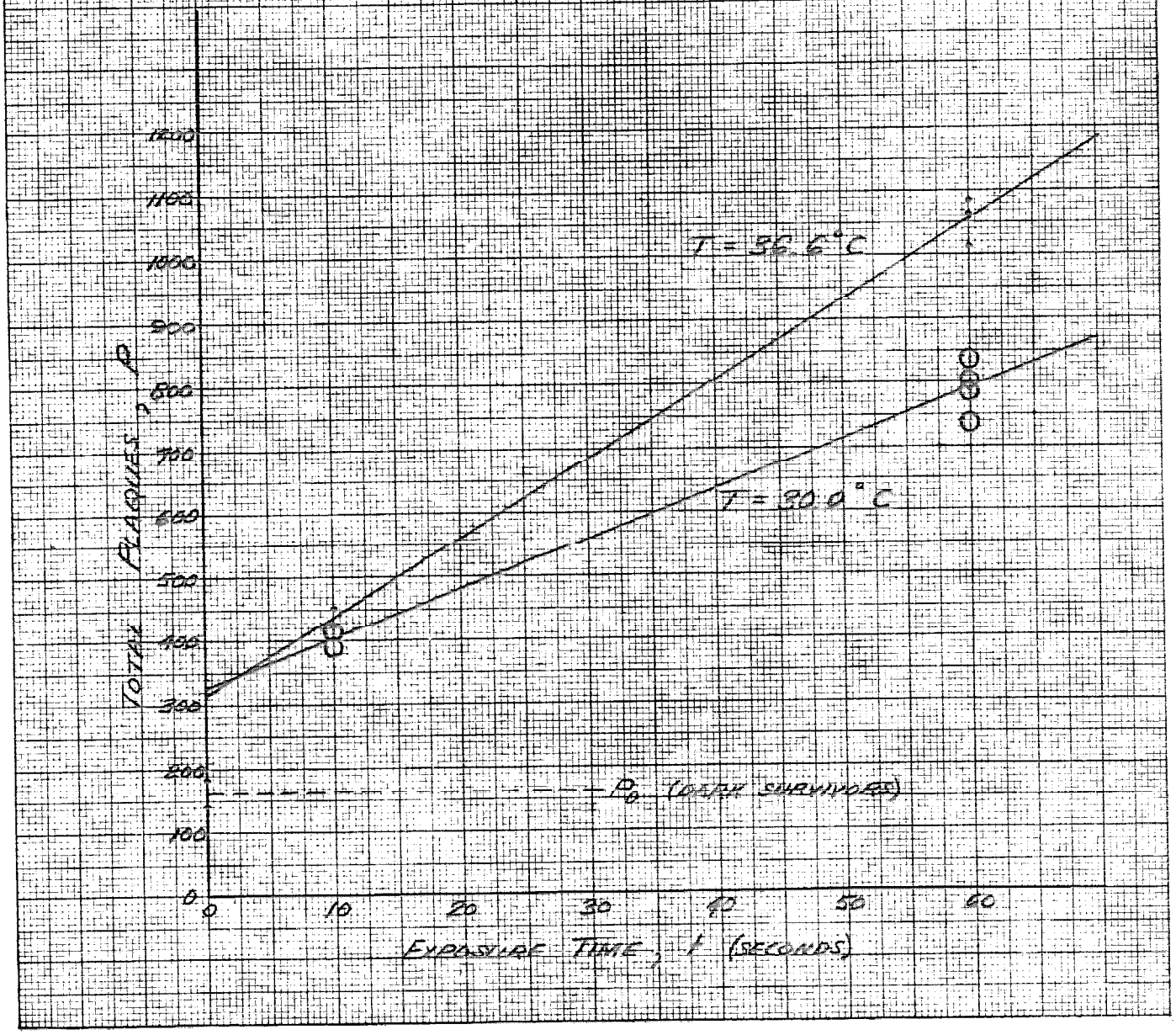


FIGURE 25: MAXIMUM PHR RATE AT 29.9°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY $I = 100$

$$\frac{R(1.0, 29.9^\circ)}{R(1.0, 36.6^\circ)} = 0.76$$

$$\therefore \frac{R_{max}(29.9^\circ)}{R_{max}(36.6^\circ)} = \left(\frac{1.02}{1.03}\right)(0.76) = 0.75$$

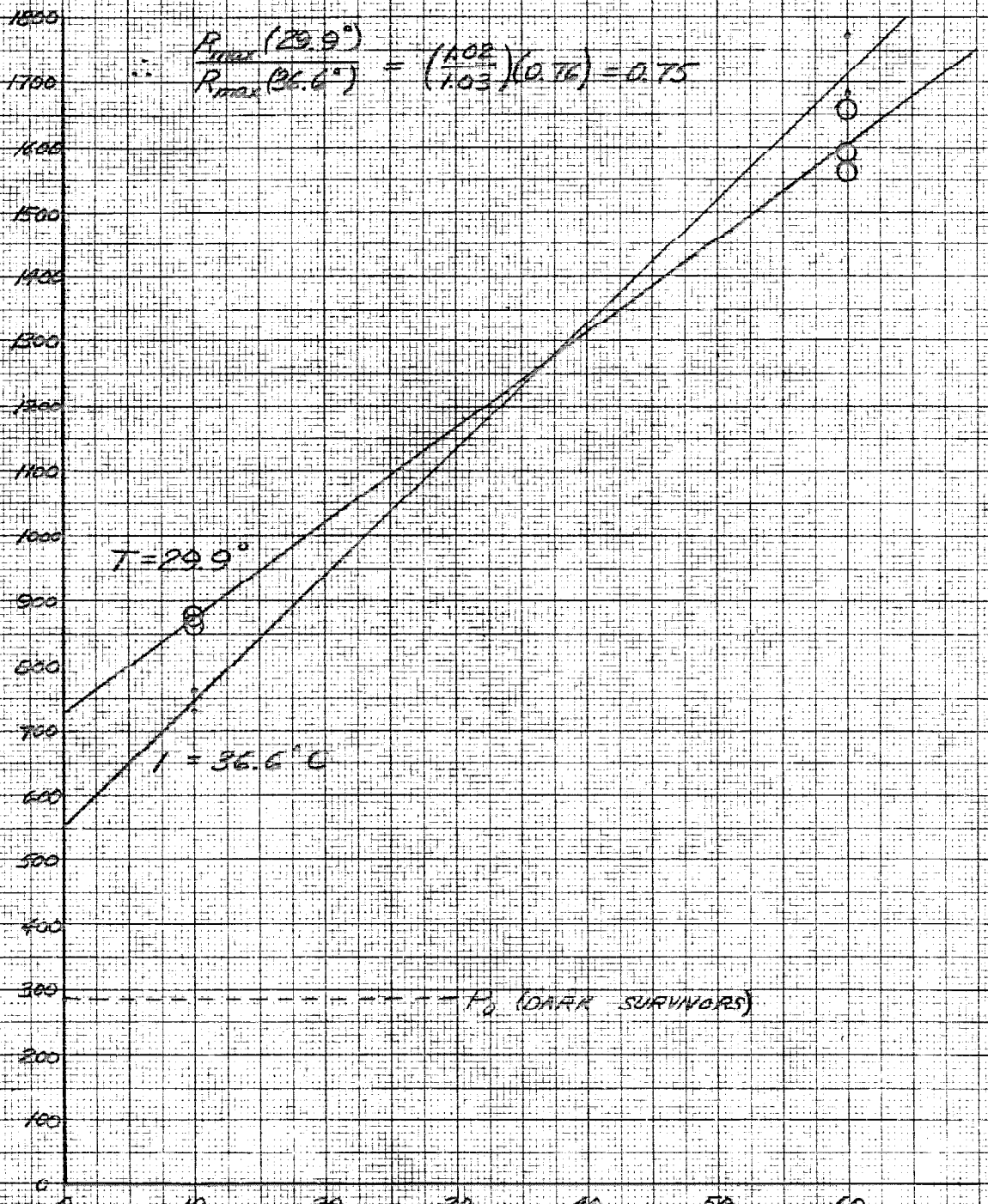


FIGURE 2C. MAXIMUM PHR RATE AT 23.2°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY $I = 100$

$$\frac{R(10, 23.2^\circ)}{R(10, 36.6^\circ)} = 0.47$$

$$\therefore \frac{R_{max}(23.2^\circ)}{R_{max}(36.6^\circ)} = \left(\frac{1015}{1405}\right)(0.47) = 0.46$$

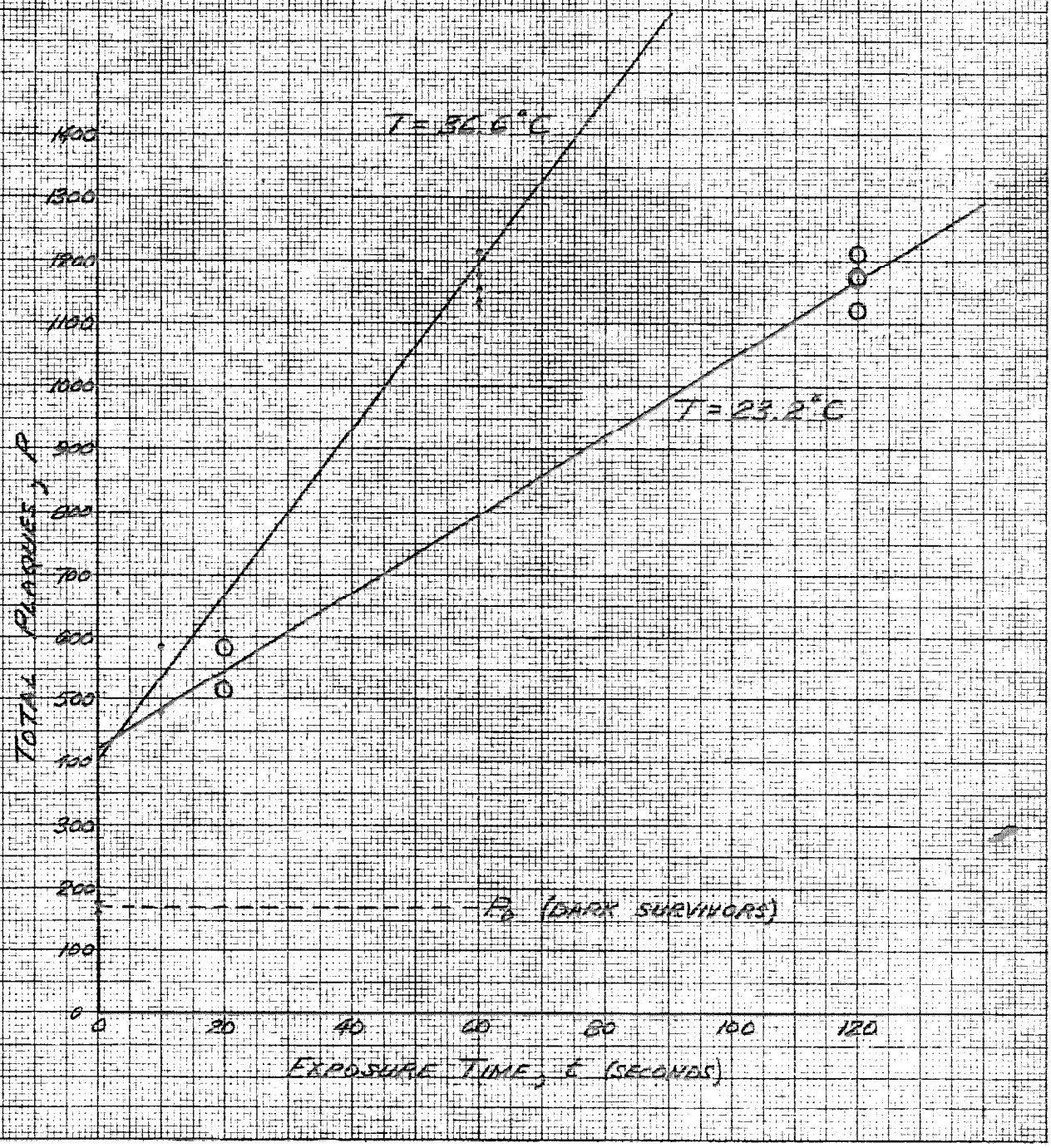


FIGURE 27: MAXIMUM PHR RATE AT 14.9°C

SINGLE LIGHT FLASH OF LENGTH $\hat{t}$, INTENSITY $I = 1.00$

$$\frac{R(1.0, 14.9^\circ)}{R(1.0, 36.6^\circ)} = 0.197$$

$$\therefore \frac{R_{max}(14.9^\circ)}{R_{max}(36.6^\circ)} = \left(\frac{1.003}{1.03}\right)(0.197) = 0.192$$

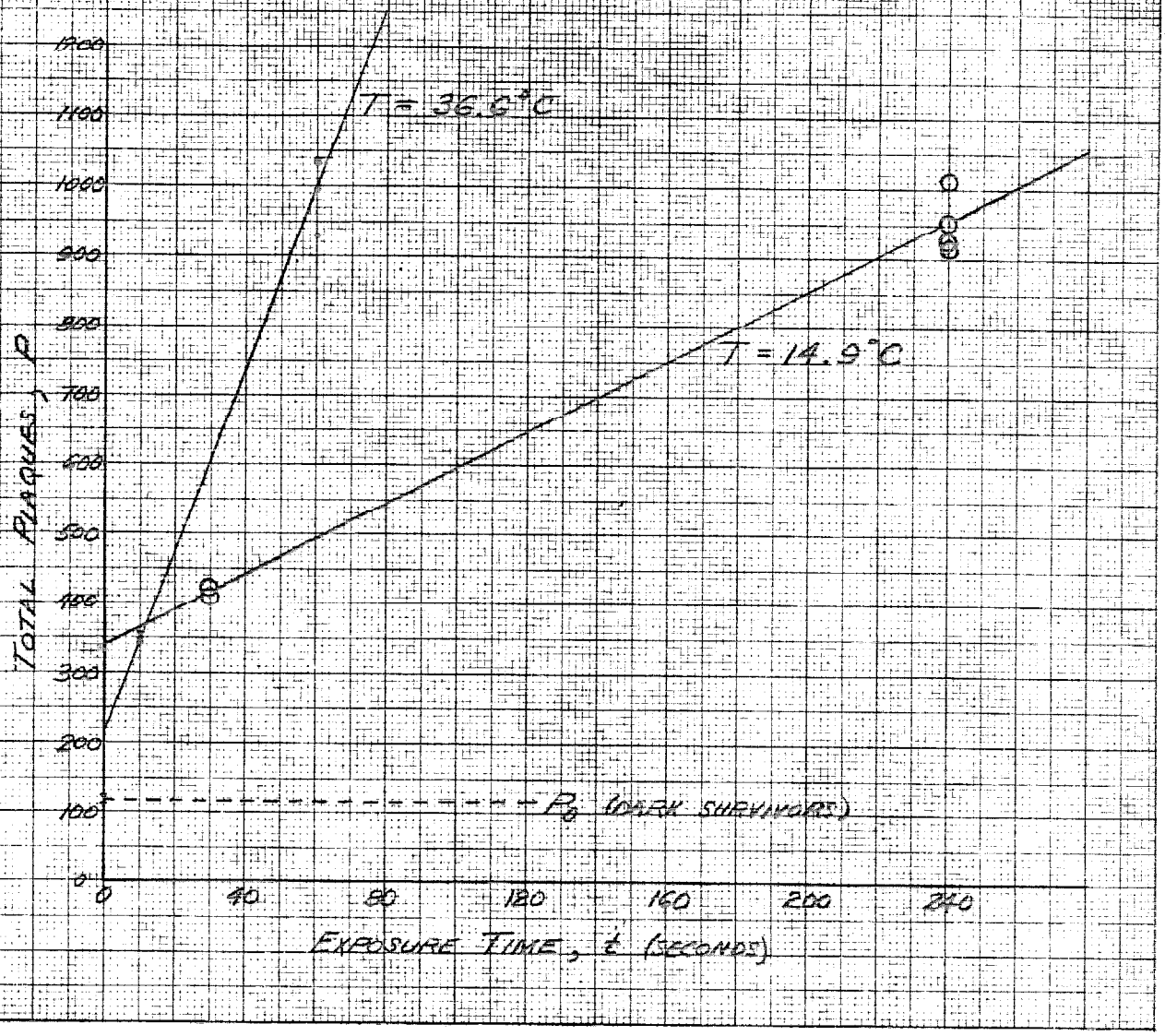


FIGURE 28. MAXIMUM PHR RATE AT 7.8 °C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY $I = 100$

$$\frac{R(1.0, 7.8°)}{R(1.0, 36.6°)} = 0.071$$

$$\therefore \frac{R_{max}(7.8°)}{R_{max}(36.6°)} = \left(\frac{0.997}{1.03}\right)(0.071) = 0.069$$

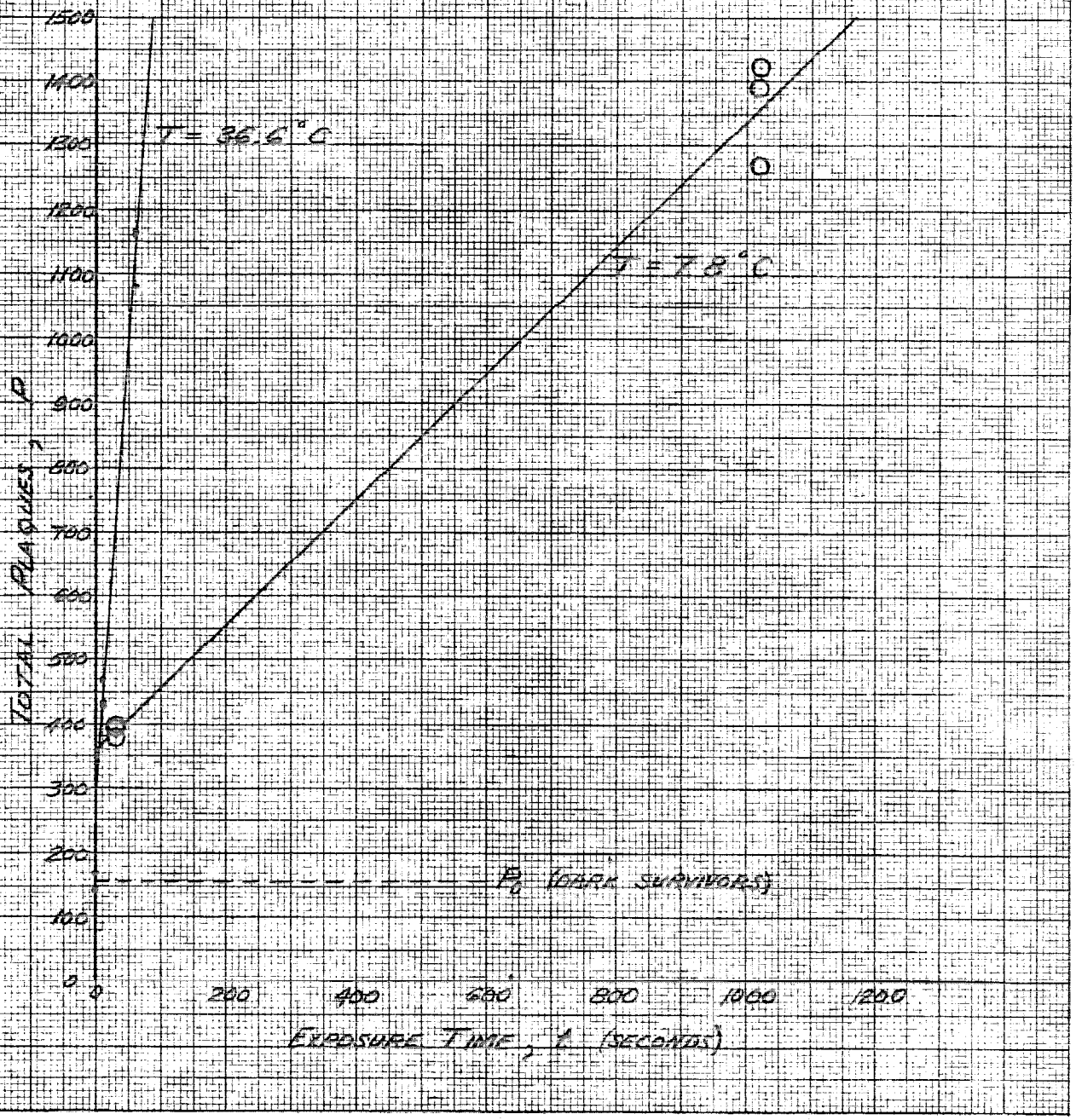


FIGURE 29: MAXIMUM PHP RATE AT 0.0°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY $I = 100$

$$\frac{R(100, 0.0°)}{R(100, 37.8°)} = 0.0187$$

$$\therefore \frac{R_{max}(0.0°)}{R_{max}(37.8°)} = \left(\frac{1.000}{1.037}\right)(0.0187) = 0.0183$$

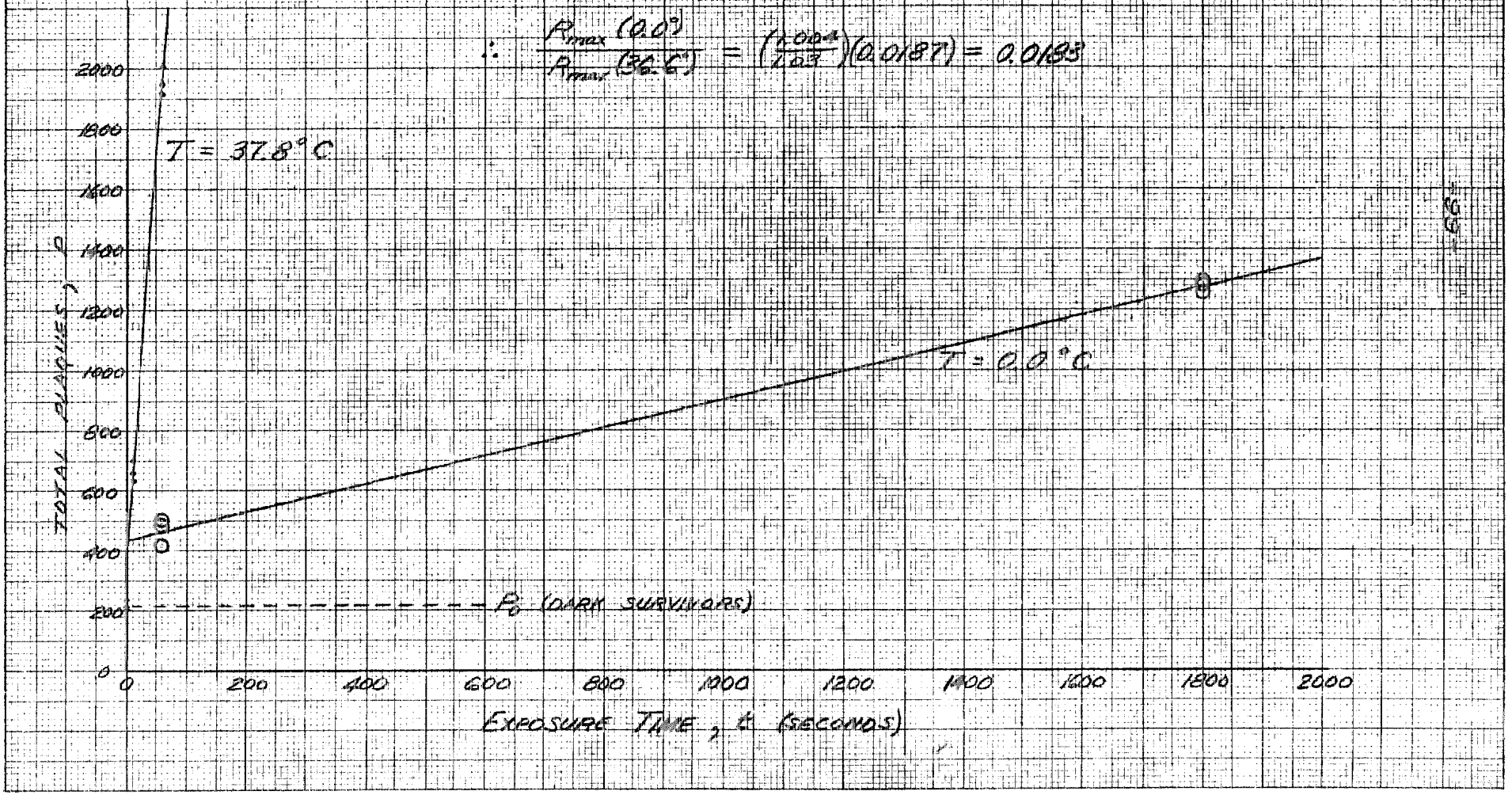
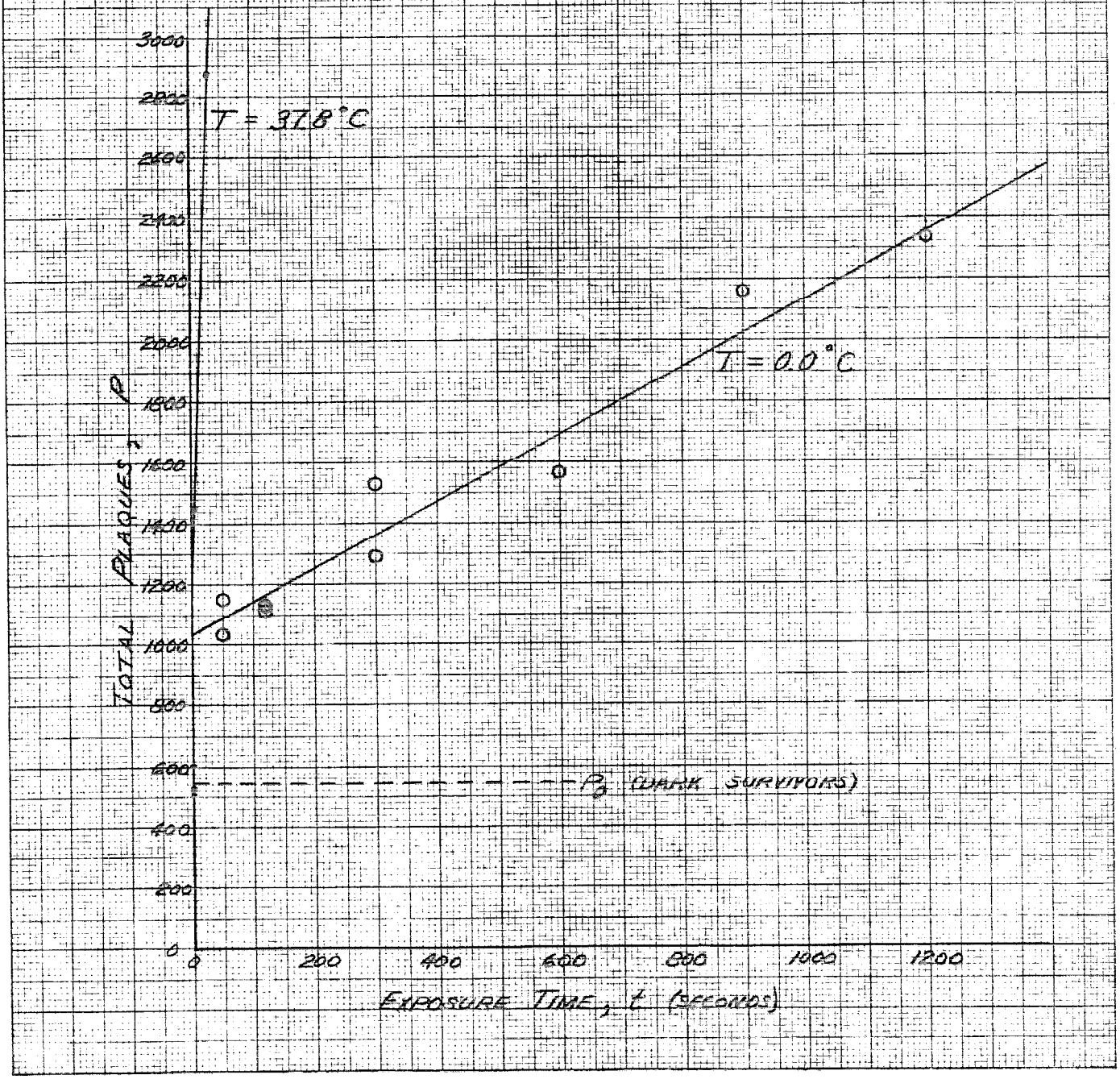


FIGURE 30: MAXIMUM PLR RATE AT 0.0°C

SINGLE LIGHT FLASH OF LENGTH t, INTENSITY $I = 100$

$$\frac{R(10, 0.0°)}{R(10, 37.8°)} = 0.0193$$

$$\therefore \frac{R_{max}(0.0°)}{R_{max}(37.6°)} = \left(\frac{1.004}{103}\right)(0.0193) = 0.0188$$



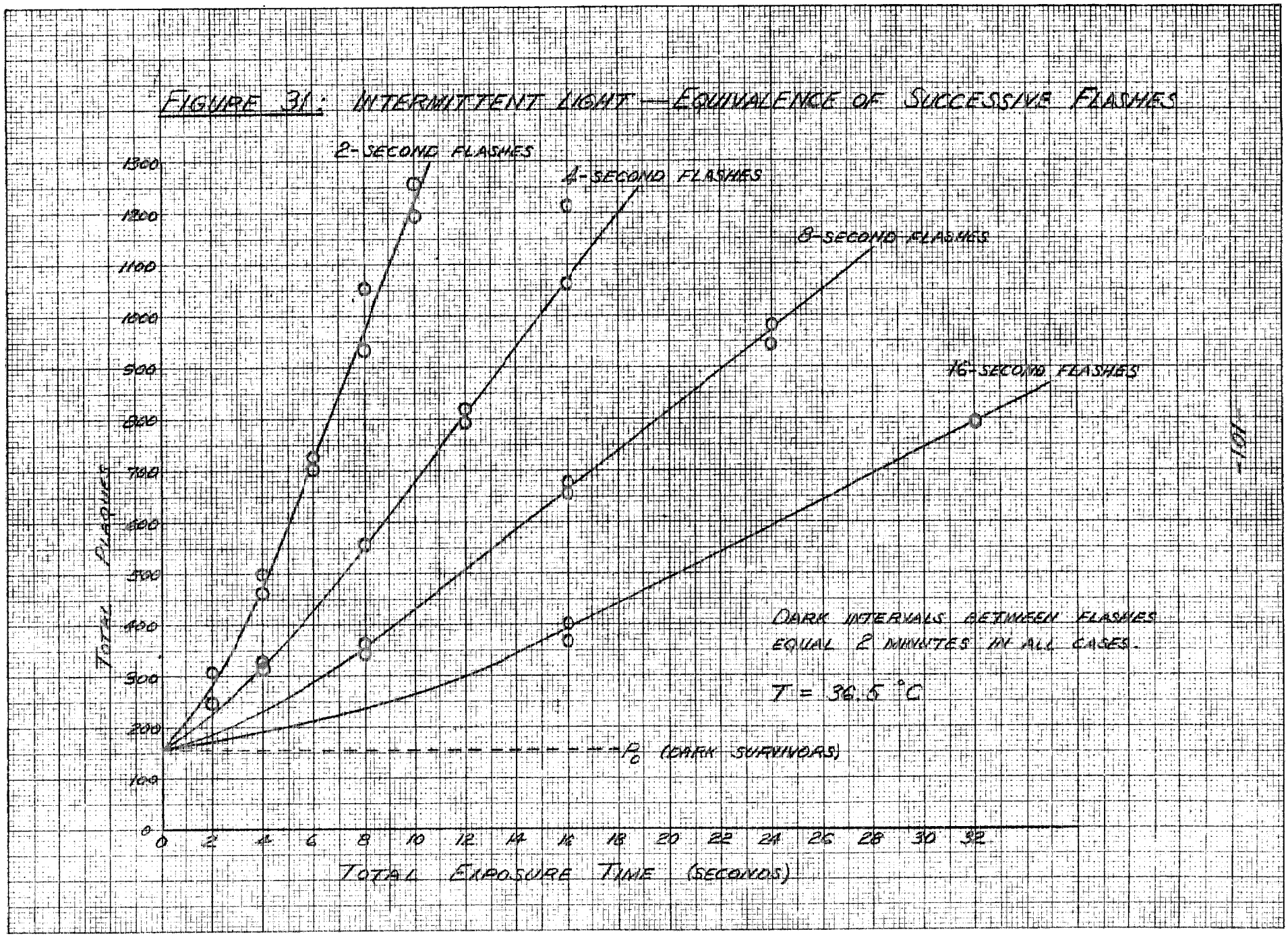
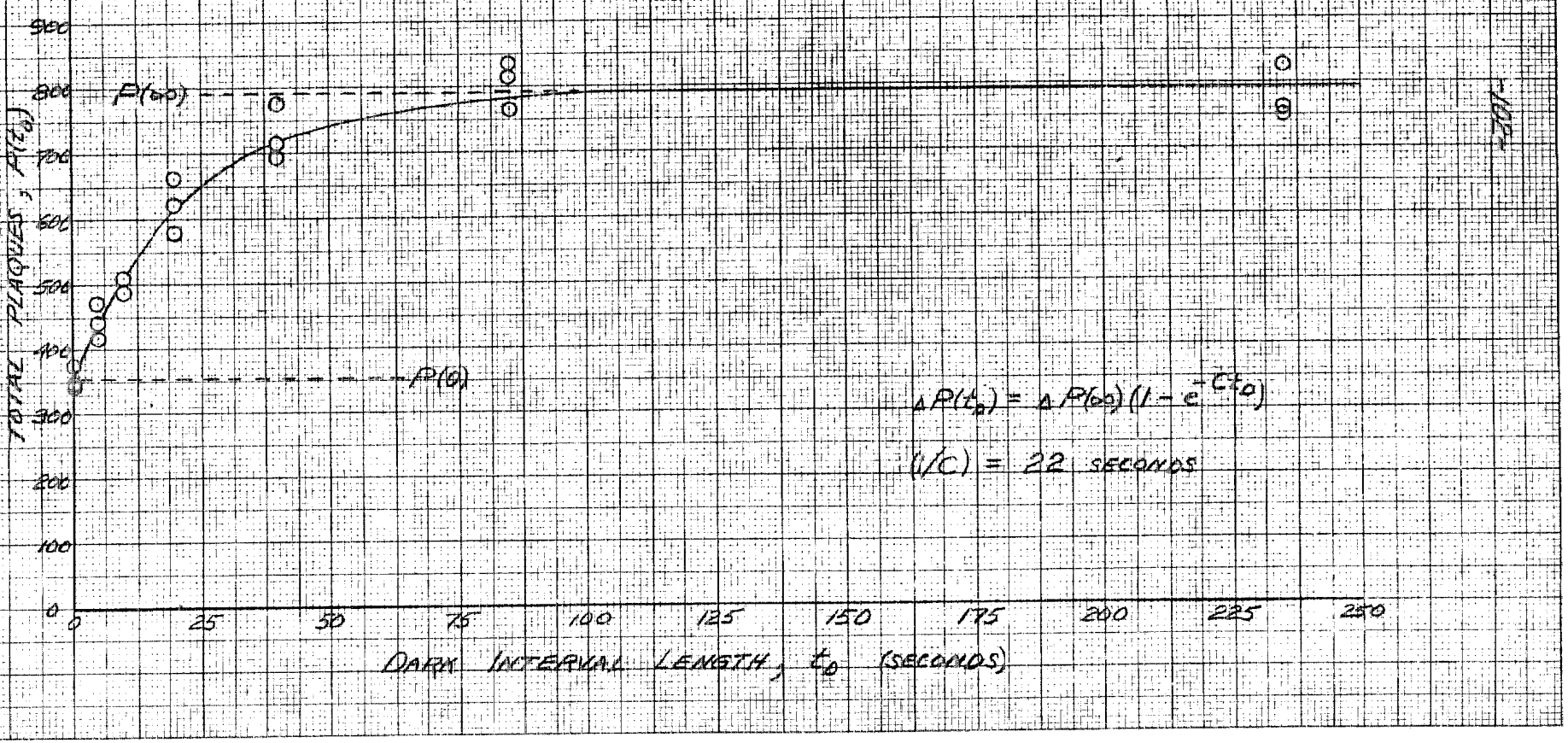


FIGURE 31: INTERMITTENT LIGHT — EQUIVALENCE OF SUCCESSIVE FLASHES
2-SECOND FLASHES
4-SECOND FLASHES
8-SECOND FLASHES
16-SECOND FLASHES
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
TOTAL PLAQUES
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
TOTAL EXPOSURE TIME (SECONDS)
DARK INTERVALS BETWEEN FLASHES EQUAL 2 MINUTES IN ALL CASES.
T = 36.5 °C
P_0 (DARK SURVIVORS)
-101-

FIGURE 32: INTERMITTENT LIGHT — EFFECT OF DARK INTERVAL LENGTH; $T = 45.0^{\circ}C$

THREE 5-SECOND FLASHES, $I = 10$, SEPARATED BY DARK INTERVALS OF LENGTH t_0



$$\Delta P(t_0) = \Delta P(60)(1 - e^{-C t_0})$$

$(1/C) = 22$ SECONDS

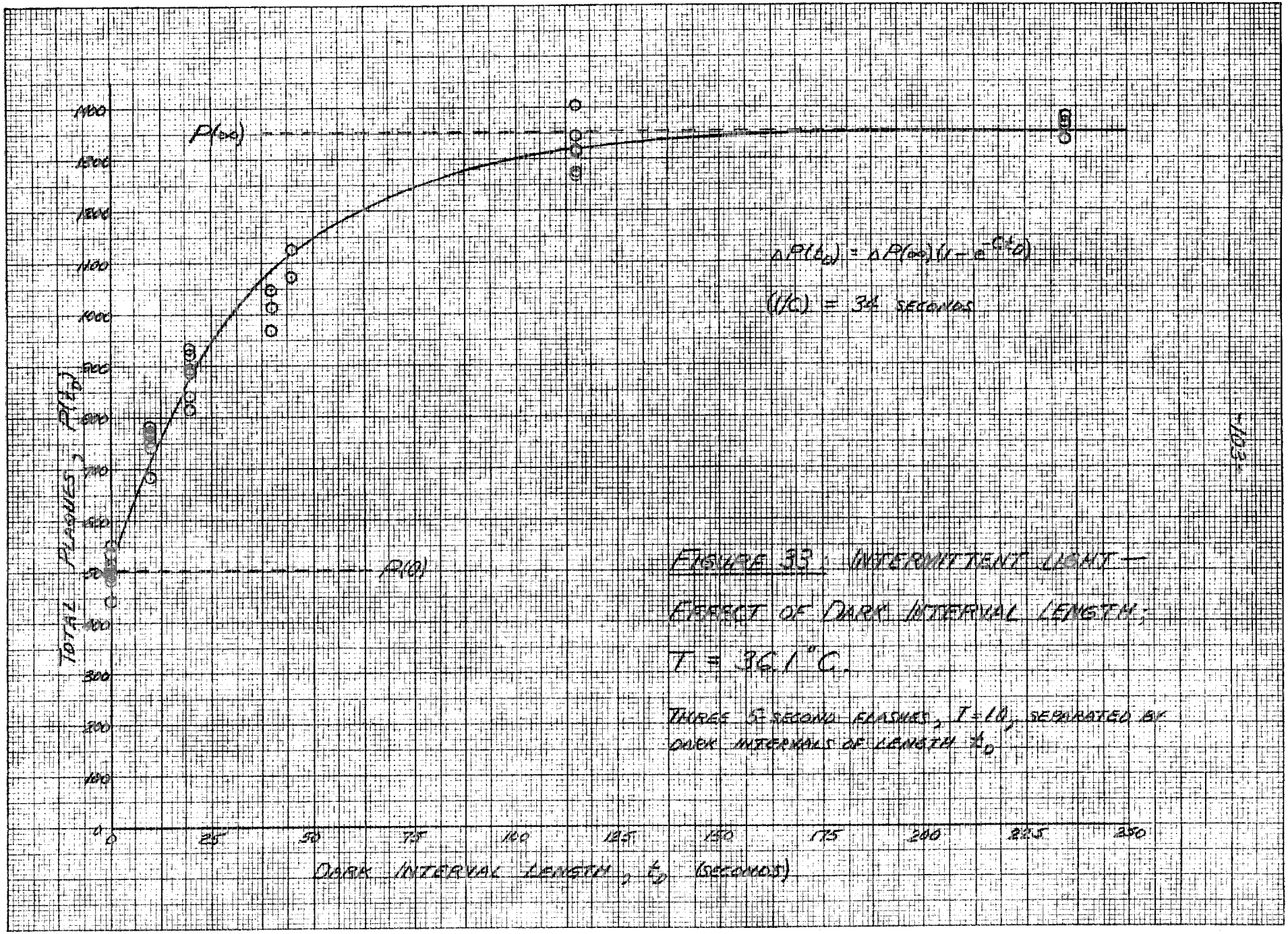


FIGURE 33. INTERMITTENT LIGHT — EFFECT OF DARK INTERVAL LENGTH; T = 96.1°C.

THREE 5-SECOND FLASHES, I = 10, SEPARATED BY DARK INTERVALS OF LENGTH t_D

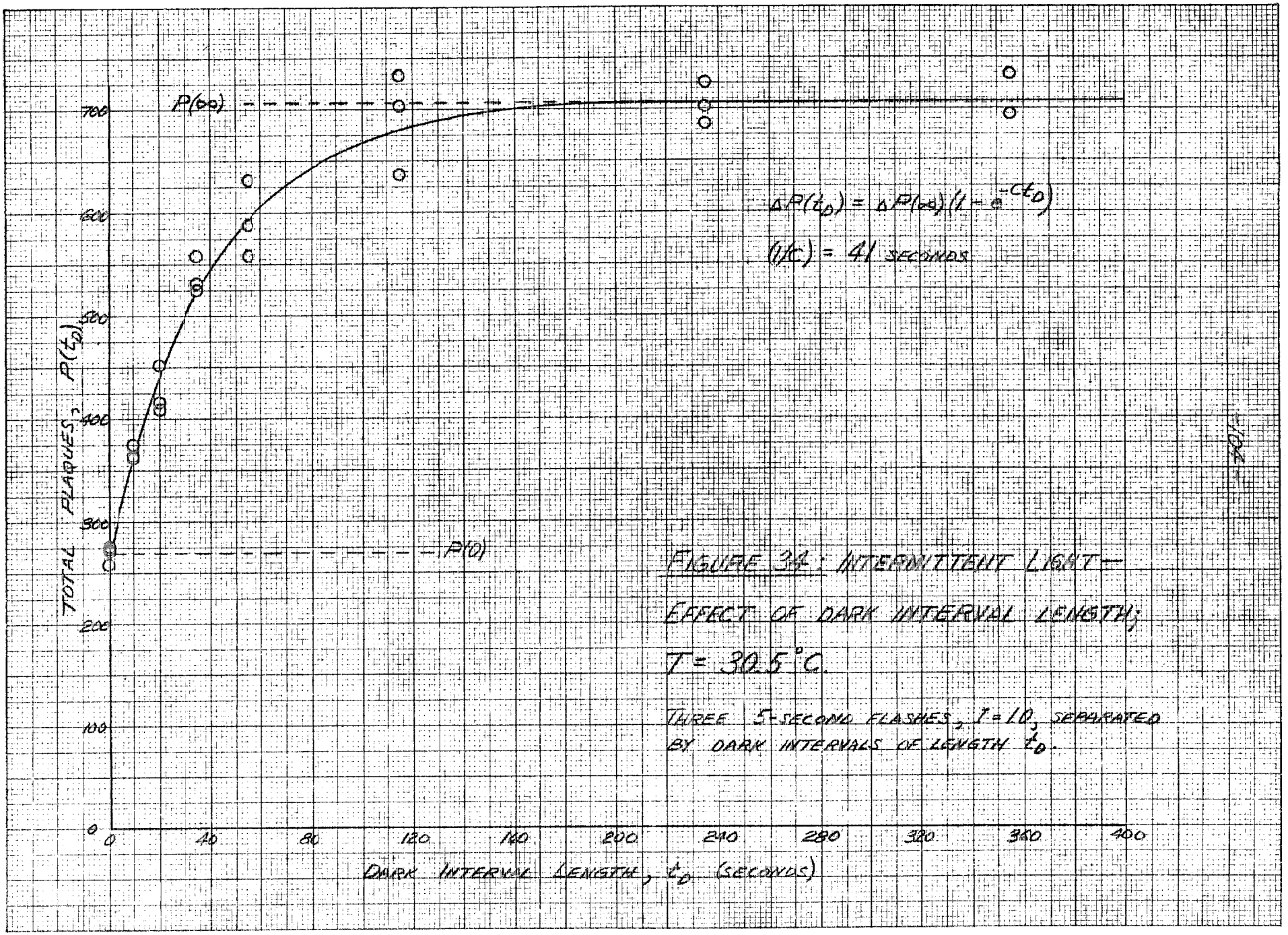


FIGURE 34: INTERMITTENT LIGHT — EFFECT OF DARK INTERVAL LENGTH; T = 32.5°C.

THREE 5-SECOND FLASHES, I = 10, SEPARATED BY DARK INTERVALS OF LENGTH t_D.

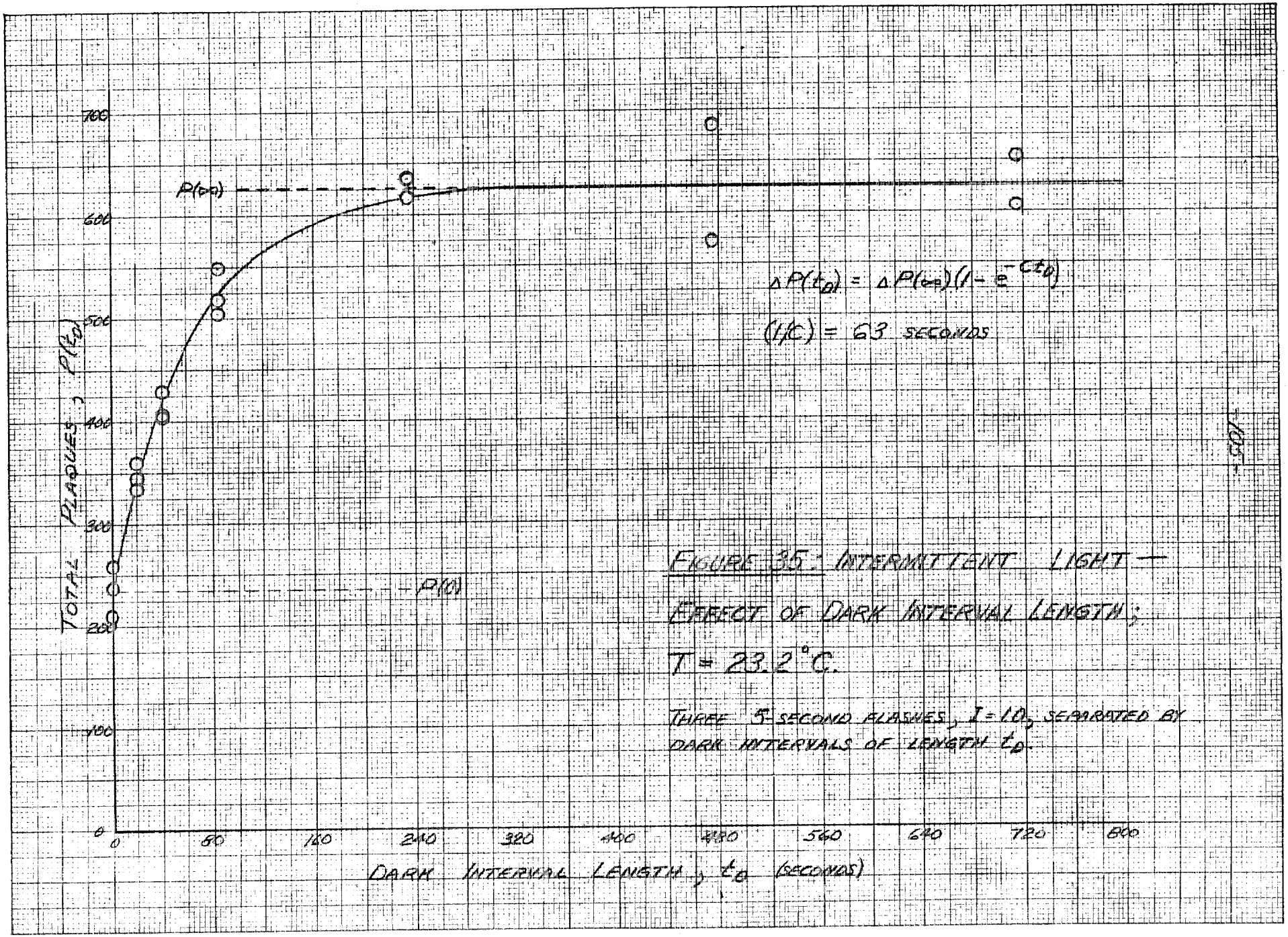


FIGURE 35: INTERMITTENT LIGHT — EFFECT OF DARK INTERVAL LENGTH; $T = 23.2^{\circ}C.$

THREE 5-SECOND FLASHES, $I = 10$, SEPARATED BY DARK INTERVALS OF LENGTH t_D.

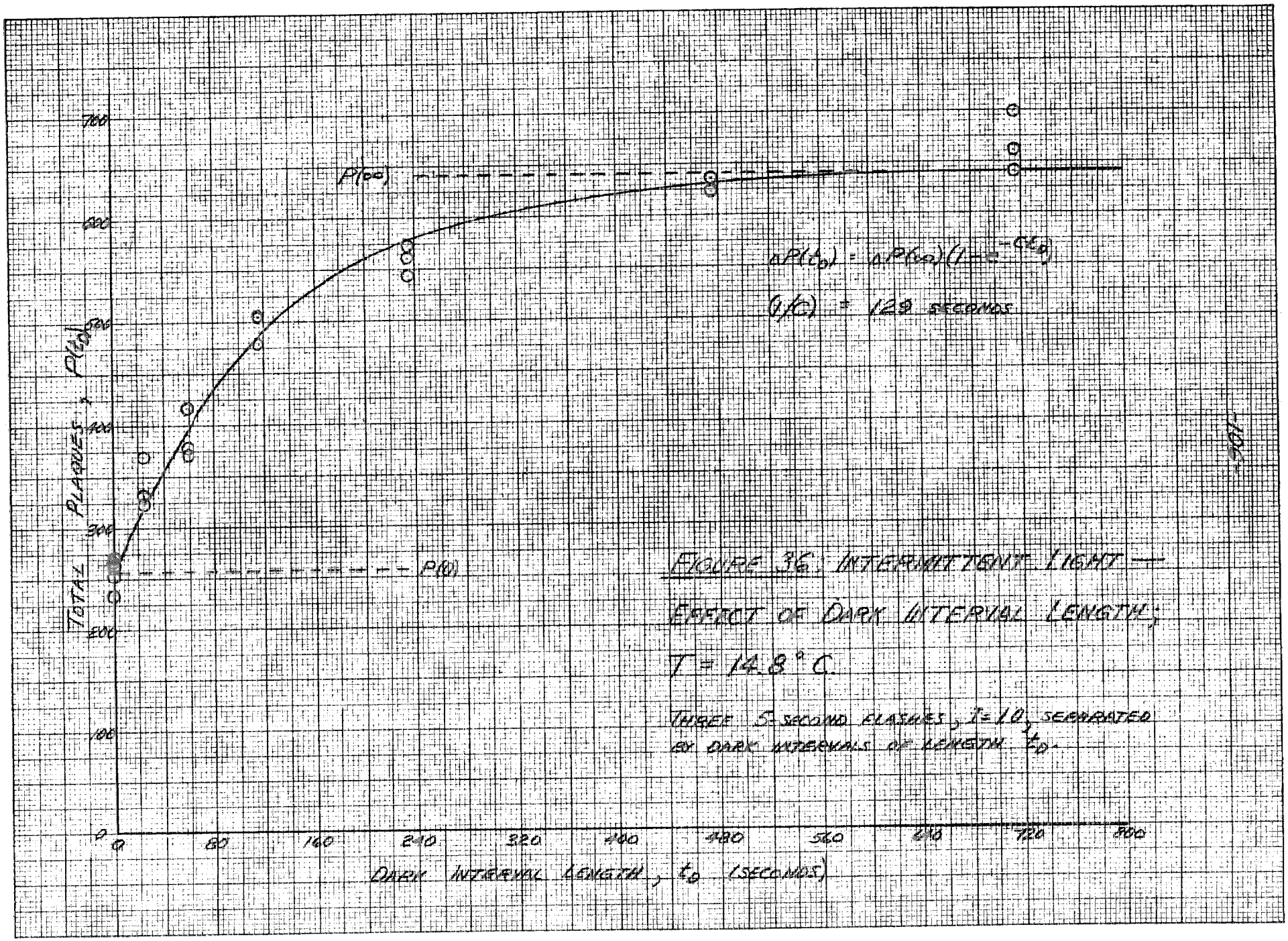


FIGURE 36: INTERMITTENT LIGHT — EFFECT OF DARK INTERVAL LENGTH; T = 14.8° C.

THREE 5-SECOND FLASHES, I = 10, SEPARATED BY DARK INTERVALS OF LENGTH t_D.

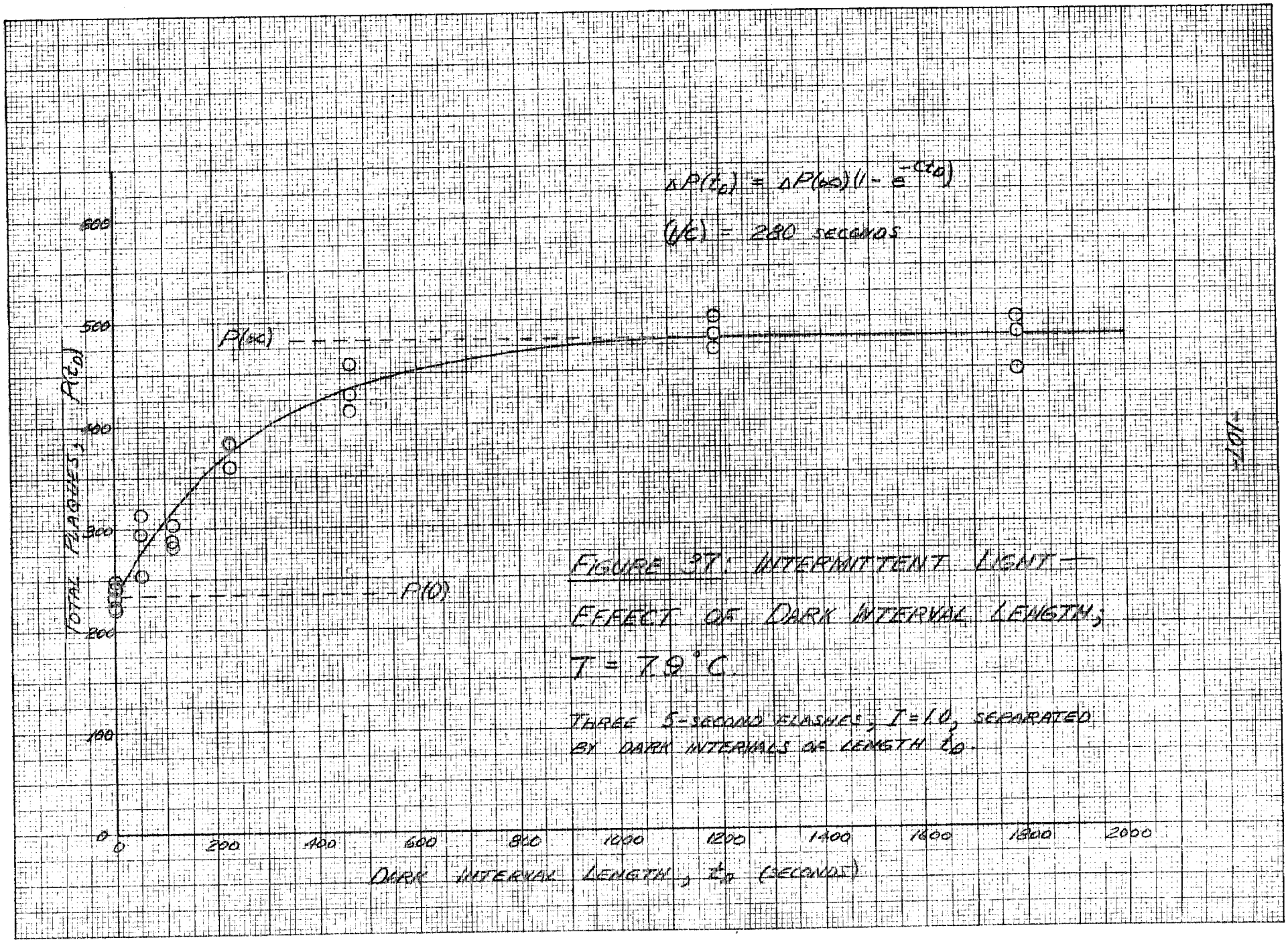


FIGURE 37: INTERMITTENT LIGHT — EFFECT OF DARK INTERVAL LENGTH; $T = 78°C$.

THREE 5-SECOND FLASHES, $T = 10$, SEPARATED BY DARK INTERVALS OF LENGTH τ_0.

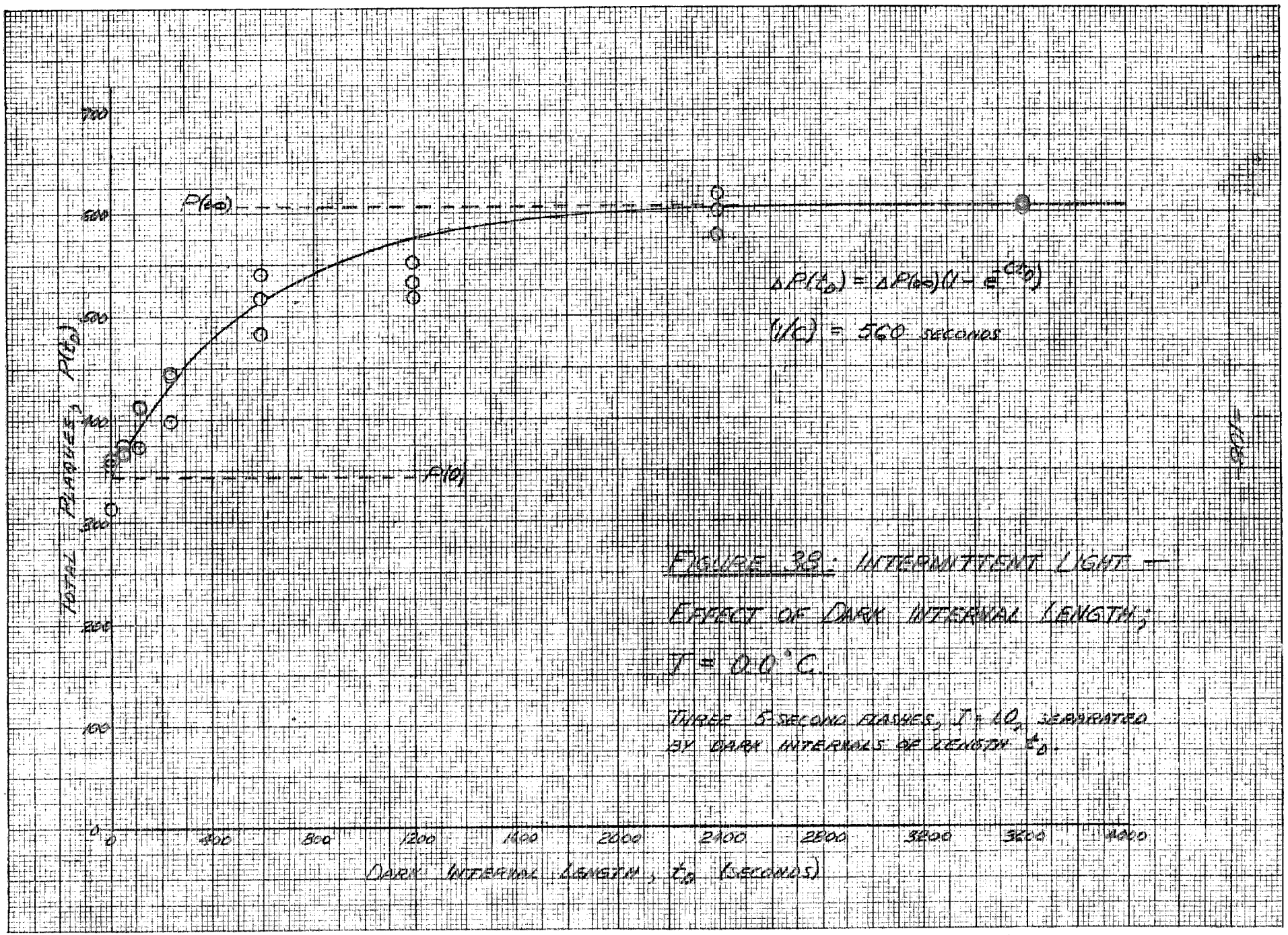


FIGURE 29: INTERMITTENT LIGHT — EFFECT OF DARK INTERVAL LENGTH; $T = 0.0\,^{\circ}C$.

THREE 5-SECOND FLASHES, $T = 0.0\,^{\circ}C$, SEPARATED BY DARK INTERVALS OF LENGTH t_D.

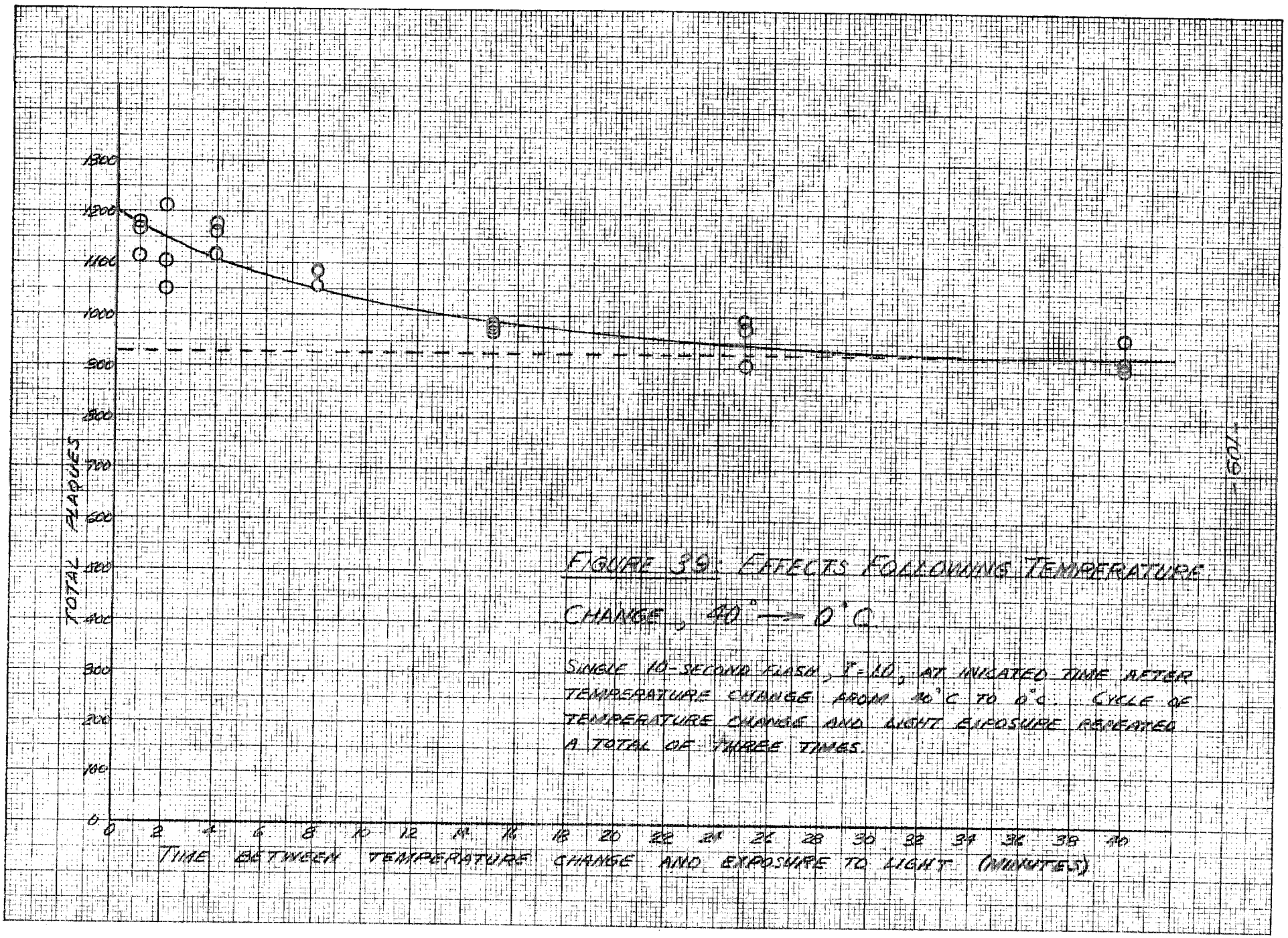


FIGURE 39: EFFECTS FOLLOWING TEMPERATURE CHANGE, 40° → 0°C.

SINGLE 10-SECOND FLASH, $I = 10$, AT INDICATED TIME AFTER TEMPERATURE CHANGE FROM 40°C TO 0°C. CYCLE OF TEMPERATURE CHANGE AND LIGHT EXPOSURE REPEATED A TOTAL OF THREE TIMES.

FIGURE 40. DEVELOPMENT OF ABILITY TO PHOTOREACTIVATE DURING ADSORPTION

SINGLE 5-SECOND FLASH, $I = 10$, STARTING AT INDICATED TIME AFTER MIXING OF PHAGE AND BACTERIA. $T = 36.5°C$. $[B] = 1 \times 10^8 \cdot ml^{-1}$. POINTS PLOTTED AT TIME ZERO ARE CONTROLS WHICH RECEIVED NO LIGHT.

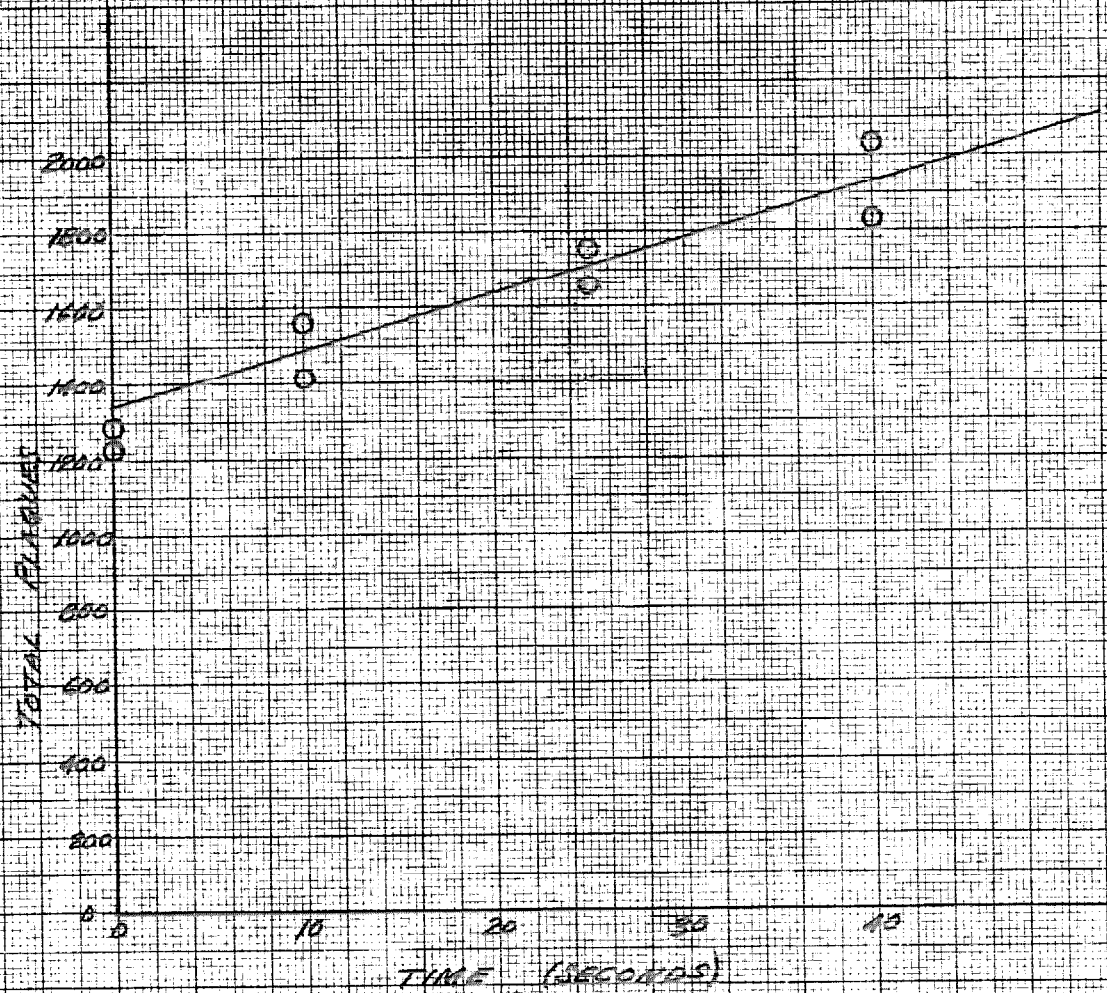
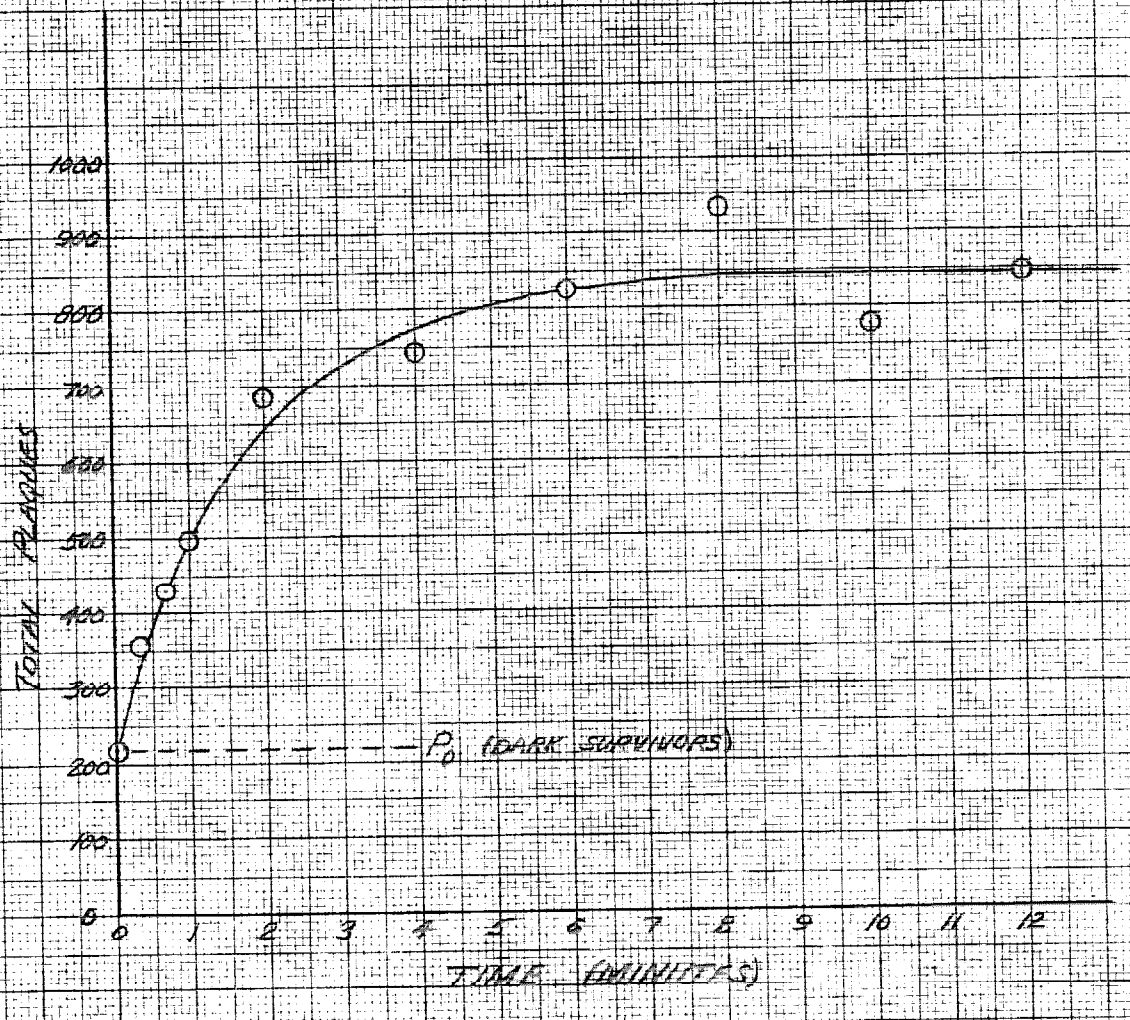


FIGURE 41: DEVELOPMENT OF ABILITY TO PHOTOREACTIVATE DURING ADSORPTION.

SINGLE 6-SECOND FLASH, $I = 1.0$, STARTING AT INDICATED TIME AFTER MIXING OF PHAGE AND BACTERIA. $T = 36.5°C$. $[B] = 1 \times 10^9 \cdot ml^{-1}$.



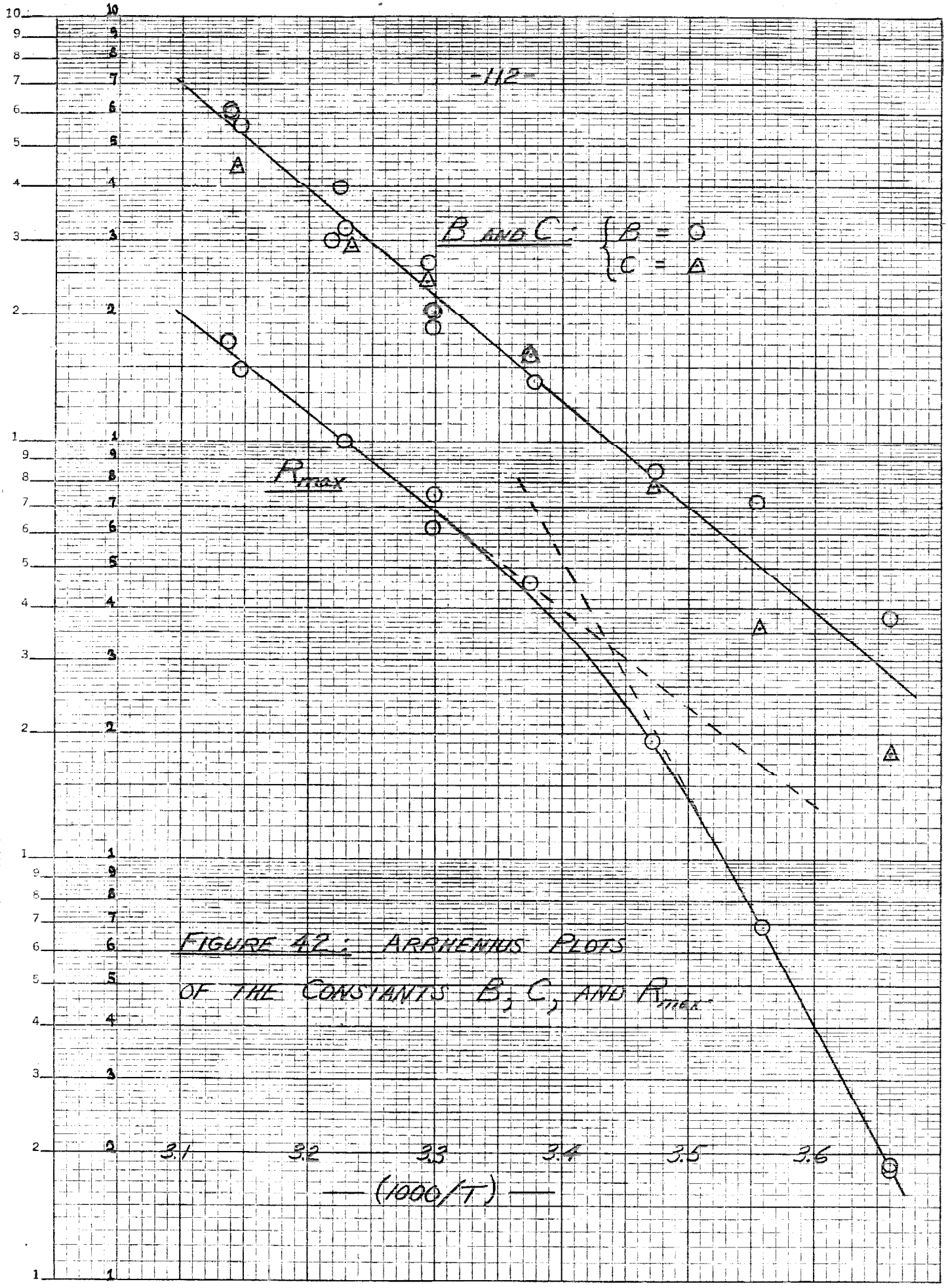


FIGURE 42: ARRHENIUS PLOTS OF THE CONSTANTS B, C, AND R_{max}.

FIGURE 43. ACTION SPECTRUM FOR PHR OF E. COLI, B/r. REDRAWN AFTER KELNER (28).

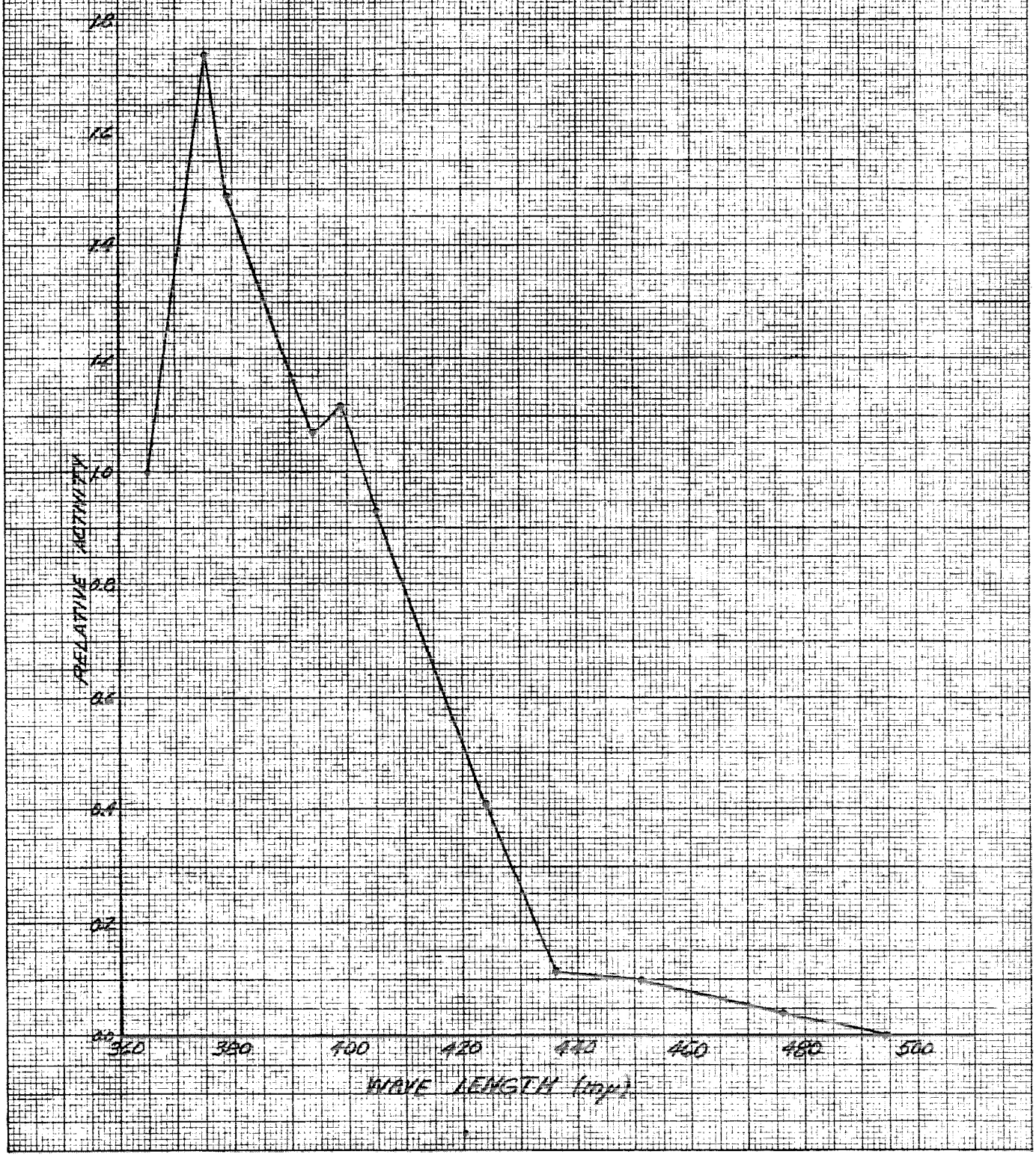


Table 1.

Summary of values of the constant B determined in the experiments of figures 7-21.

The rate, R, of PhR at light intensity I is given by the function

$$R = R_{max} \left(\frac{I}{I+B}\right) .$$

B is given in arbitrary units of light intensity relative to the standard intensity of 1.0.

Data from figure number	Temperature	B
7	45.5° C	0.061
8	45.4	0.060
9	44.7	0.056
10	37.6	0.030
11	37.0	0.040
12	36.6	0.032
13	30.4	0.0265
14	30.1	0.0205
15	30.0	0.0186
16	29.9	0.0202
17	23.2	0.0160
18	22.8	0.0139
19	14.7	0.0005
20	8.2	0.0072
21	0.0	0.0038

Table 2.

Summary of values of the constant R_{max} determined in the experiments of figures 22-30.

The rate, R, of PhR at light intensity I is given by the function

$$R = R_{max} \left(\frac{I}{I+B}\right) .$$

R_{max} is given in arbitrary units relative to R_{max} at 36.6° C, which is defined as 1.0.

Data from figure number	Temperature	R_{max}
22	45.7° C	1.72
23	44.7	1.49
--	36.6	1.00 (definition)
24	30.0	0.62
25	29.9	0.75
26	23.2	0.46
27	14.9	0.192
28	7.8	0.069
29	0.0	0.0183
30	0.0	0.0188

Table 3.

Summary of values of the constant C determined in the experiments of figures 32-38.

The increase in plaque count, $\Delta P(t_D)$, with increase in the length, t_D, of the dark intervals separating the light flashes of a series of identical flashes is given by the function

$$\Delta P(t_D) = \Delta P(\infty)(1 - e^{-Ct_D}).$$

(1/C) is given in units of time and is the time constant of the increase in plaque count.

Data from figure number	Temperature	(1/C)	C
32	45.0° C	22 sec	0.045 sec^{-1}
33	36.1	34	0.029
34	30.5	41	0.024
35	23.2	63	0.016
36	14.8	129	0.0078
37	7.9	280	0.0036
38	0.0	564	0.0018

Appendix

The approximation of first-order reactivation kinetics by a multiple-hit mechanism.

If every phage particle which is capable of PhR were reactivated by a single hit, or event, with the same probability per unit time, then the increase in active particles with time of exposure to light would be given by

$$\Delta P = \Delta P_{max} (1 - e^{-at}) \qquad (1a)$$

$$\ln(1 - \frac{\Delta P}{\Delta P_{max}}) = -at \; . \qquad (1b)$$

A plot of $\ln(1 - \Delta P/\Delta P_{max})$ *vs.* t would then give a straight line of slope (-a) passing through the origin. PhR of phage T2 approximately, but not exactly fulfills this condition.

If each particle receives a number of inactivating hits and PhR occurs by direct point-by-point repair of these damages, then in general the first-order kinetics indicated by equations (1a) and (1b) will not be followed. However, under certain conditions multiple-hit reactivation many closely approximate first-order kinetics over a small range of ultraviolet doses. It is possible that the observed PhR kinetics of T2 may arise in this way.

For simplicity it will be assumed that the probability of survival per phage particle is $p = e^{-N}$, where N is the

average number of damages per particle. This is a satisfactory approximation to the ultraviolet survival of T2, since we are concerned only with effects at relatively large ultraviolet doses. Let $N = m + n$, where m is the mean number of repairable damages produced per particle by the ultraviolet irradiation.

If all the repairable hits are repaired at the same rate during PhR, then the total number of damages remaining after time t of exposure to light will be

$$N = n + me^{-at}, \tag{20}$$

and therefore

$$(1 - \frac{\Delta P}{\Delta P_{max}}) = (\frac{1 - e^{-me^{-at}}}{1 - e^{-m}}) . \tag{21}$$

In figure 44 this function is plotted on a logarithmic scale against for m = 1, 2, 3, 4, and 5. The value m = 4 corresponds approximately to the ultraviolet dose usually used in our PhR experiments. All of the curves of figure 44 are strongly "multiple-hit" in character, i.e. show considerable curvature near the origin, and clearly do not agree with the experimental results in PhR.

Suppose, however, that the repairable damages consist of two or more classes having different rates of repair during light exposure. Qualitatively the result will be that the most quickly repaired types of damage will disappear first, leading to a decrease in the rate of PhR,

which may under favorable circumstances offset the increase in rate seen in the curves of figure 44. Quantitatively the results will be:

$$N = n + \sum_i m_i e^{-a_i t} \qquad (22)$$

$$(1 - \frac{\Delta P}{\Delta P_{max}}) = \frac{1 - e^{-\sum_i m_i e^{-a_i t}}}{1 - e^{-\sum_i m_i}} . \qquad (23)$$

In figure 45 three curves are shown, all calculated for a total of 4 hits: $\sum_i m_i = 4$. The curves are for: (A) all damages repaired at an equal rate; (B) two equal classes of damages repaired at relative rates of 1:4; (C) four equal classes of damages repaired at relative rates of 1:2:4:8. The rapid approach of the curves to the one-hit curve of equation (1b) is apparent. Also plotted in figure 45 are the experimental data of figure 3, with the time scale adjusted to give the same ultimate slope as the calculated curves. The data fit curve (C) very well, illustrating that the observed kinetics of PhR can be accounted for along the lines suggested.

The shape of the curve calculated on the basis of any particular assumed distribution does change somewhat with the value of m, i.e. with the ultraviolet dose. In figure 46 curves are shown which were calculated for $m = \sum_i m_i = 2$, 3, 4, 5, and 6 using the same distribution as for curve (C)

of figure 45. A critical experimental test for such changes in the PhR curve would be difficult, however, because work is confined to such a narrow range of conditions. The complication of multiplicity reactivation makes experiments impossible at survivals lower than about 10^{-4}, which corresponds to about m = 5. On the other hand, at m = 2, the plaque count at maximum PhR is only seven times the count of survivors before PhR. At this, or at smaller ultraviolet doses the total change is so small that it would be difficult to obtain the shape of the curve with accuracy. Finally it is to be remembered that the experimental values of $(1 - \Delta P/\Delta P_{max})$ are obtained as the difference of two numbers, and that the plaque counts cannot be obtained accurately enough to make this difference accurate when it is small. Thus only the first part of the curve can be measured satisfactorily in any case.

FIGURE 44.

$$\text{LOG}\left(\frac{1-e^{-mc^{-at}}}{1-e^{-m}}\right) \quad \text{VS.} \quad (at)$$

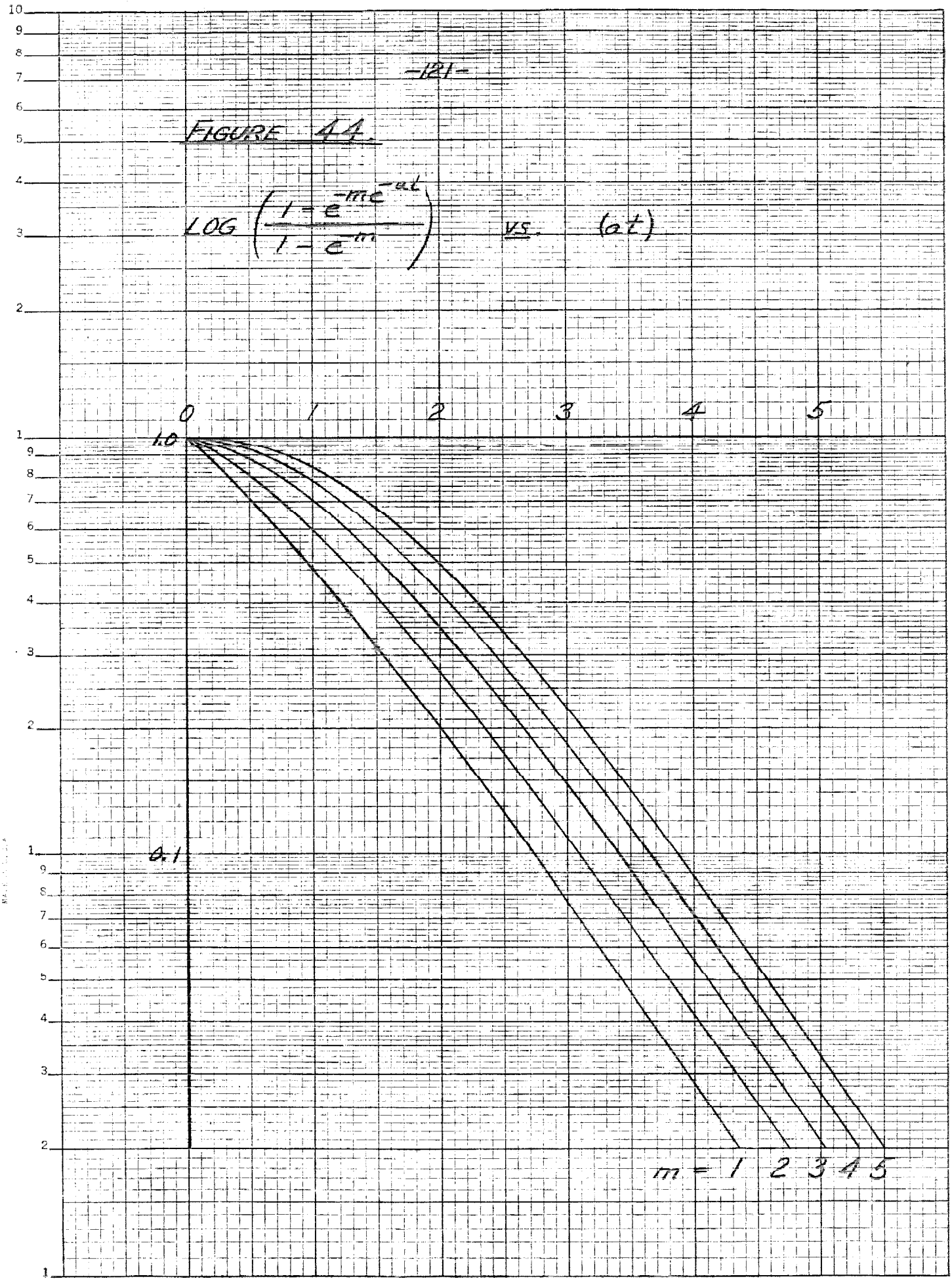


FIGURE 45.

$$\log\left(\frac{1-e^{-\sum_i m_i e^{-q_i t}}}{1-e^{-\sum_i m_i}}\right) \text{ vs } (at)$$

SEE TEXT FOR DESCRIPTION OF CURVES.

○ = EXPERIMENTAL POINTS FROM FIGURE 3.

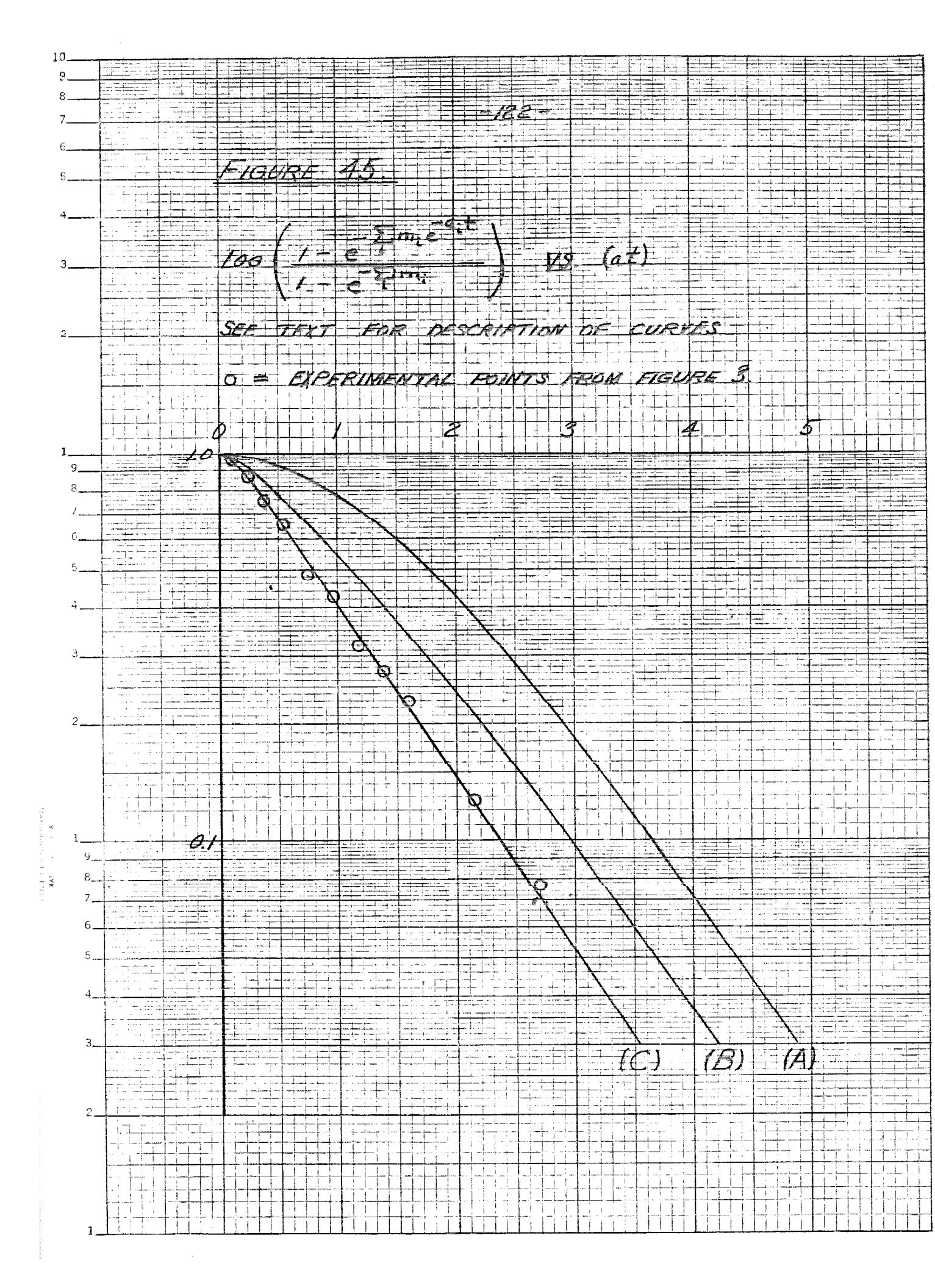


FIGURE 46.

$$\text{LOG}\left(\frac{1-e^{-\sum_i m_i e^{-a_i t}}}{1-e^{-\sum_i m_i}}\right) \text{ VS } (a_i t)$$

SAME DISTRIBUTION OF m_i AND a_i AS CURVE (C) OF FIGURE 45.