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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

CHANGES IN RADIORESISTANCE ASSOCIATED WITH CHANGES IN METABOLISM IN *STAPHYLOCOCCUS AUREUS*

A DISSERTATION

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DOCTOR OF PHILOSOPHY

BY

ROBERT DALE HOWELL

Norman, Oklahoma

1966

CHANGES IN RADIORESISTANCE ASSOCIATED WITH CHANGES

IN METABOLISM IN STAPHYLOCOCCUS AUREUS

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CHANGES IN RADIORESISTANCE ASSOCIATED WITH CHANGES IN METABOLISM IN *STAPHYLOCOCCUS AUREUS*

CHAPTER I

INTRODUCTION

Studies conducted by physicists in the late 1800's opened a new era of biology. In 1895, W. C. Roentgen discovered x-rays. This discovery was closely followed by Becquerel's discovery of natural radioactivity in 1896. Each of these two workers, unknowingly, performed biological experiments. Roentgen made visible the bones of his hand by placing it between his vacuum tube and an improvised fluorescent screen, and Becquerel is said to have burned himself with radium carried in his pocket. Roentgen's experience stimulated the development of diagnostic radiology, and Becquerel's skin damage led to an earnest medical investigation of the effects of natural radioactivity (Grosch, 1965).

In these early days pathologists and physicists were faced with many fundamental aspects, chiefly in connection with the therapeutic use of x-rays in medicine and the resulting need to study the biological action of these rays.

Because of this need for a better understanding of the biological action of radiation the area of science known as radiobiology came to be (Errera and Forssberg, 1961).

A few biological studies on the effects of radiation were conducted in the late 1800's. Minch, as early as 1896, attempted to study the bactericidal effects of x-rays, and even before this workers unknowingly made laboratory observations on the effects of radiation on bacteria (Zelle and Hollaender, 1955). From the late 1890's to the early 1940's a great deal of research in radiobiology was conducted by various investigators. A large part of this work yielded negative results. That which yielded positive results was more or less overlooked, and in many cases considered unimportant.

Except for attention by geneticists, sustained general interest in radiobiology is chiefly an aftermath of the atomic explosions of 1945. Full appreciation of even the genetic interest in radiobiology was delayed until after this significant date. Although H. G. Muller reported his convincing experiments that mutations could be induced by radiation im 1927, it was not until 1946 that public recognition came with the award of the Nobel Prize (Grosch, 1965).

Since this date there has been a tremendous expansion of research in radiation chemistry, physics, and biology. Because of this expansion a large volume of material has been published on radiation chemistry. Effects of radiation

on both organic and inorganic substances have been studied. Included are studies of the effects of radiation on molecules of biological origin (Hart and Platzman, 1961).

In vitro studies are important, and because of these a great deal of radiochemical reactions and mechanisms of energy transfer are now better understood. However, they have been of little value in understanding the mechanisms of radiation damage in vivo. One of the many examples where this has been proved to be the case is in studies which have been conducted on the effects of radiation on purified enzymes (Dale, 1940 and 1956; Barron et al., 1948; Le May, 1951; Mc Donald, 1954 and 1955; Mc Donald and Moore, 1955). These workers found that although radiation could inactivate certain enzymes in vitro, that the conditions necessary to show this inactivation were far removed from those normally found in the living cell.

Because of problems involved in relating in vitro studies to in vivo systems, workers have almost entirely turned to studies in living systems. Here again, though, this presents a problem. Radiation by definition is a physical phenomenon in which energy travels through space (Grosch, 1965). When radiation passes through matter it loses all or part of its energy, and within a very short space of time this energy brings about chemical changes. This energy loss is non-specific. M. N. Meissel (1956) summed up the situation when he remarked that there were no

"structures or substrata in living matter which would be insensitive to and not affected to some degree by irradiation." As to what structures or substrata are affected depends not only upon the type and energy of the radiation used, but also on the biological system being irradiated.

Scientists have looked for some common ground in which they might compare the radiation effects in different systems. This common ground is a study of the effects of radiation on the biochemical and genetic mechanisms of the cell. Research on the effects of radiation on biochemical mechanisms has been under way for a relatively short period of time. The main approach has been to attempt to define radiation damage in terms of interference with biochemical systems through research on irradiated animals and microorganisms. Although this type of research has yielded a vast amount of information, no satisfactory explanation of the exact mechanism by which tissue damage is inflicted has yet been obtained (Haddow, 1956).

Studies made in vivo are complicated by the existence of a great variation in the sensitivity of different tissues and organisms to radiation (Bacq and Alexander, 1961; Ord and Stocken, 1961). These complications are increased by variations in the sensitivity of the same organism. It has been known since 1903, that the response of an organism to radiation depends on the stage of its development at which the does is received (Ord and Stocken, 1961). In most cases

where stage of development is considered it is usually morphological. Technical difficulties at present restrict the variety of cells which can be studied in this manner, and it becomes very difficult if not impossible to correlate many of the findings from one system to another.

The ability to define the stage of development in most cases is very difficult. The advantages of microorganisms for studies of this type were recognized by a number of investigators in the late thirties. In the twenty-five years since the yeast Torula cremoris was used by Anderson and Turkowitz in 1941, to investigate radiosensitivity in the presence and absence of atmospheric oxygen, a substantial body of information has accumulated describing conditions under which various factors exert their influence on radiosensitivity (De Serres, 1961).

Many factors have been shown to influence the radiosensitivity of bacteria. These include temperature during growth and irradiation, growth medium, oxygen concentration, pH, moisture content, and stage of growth (Zelle and Hollaender, 1955).

Roberts and Aldous (1949) have shown that resting cells of Escherichia coli B grown in broth are less sensitive to radiation than resting cells grown in broth of the same type plus glucose. They also noted that resting cells grown in broth with a final pH of 8.0 were less sensitive than resting cells grown in broth with a final pH of 7.0.

Hollaender and Claus (1936) found that 7 hr old agar slant cultures of E. coli were more resistant to ultraviolet light than their standard 15 hr old cultures. Ten day old cultures of this organism were more sensitive than 15 hr old cultures. Demerec and Latarjet (1946) observed that cultures of E. coli B/r in the lag and stationary phase of growth were more resistant to ultraviolet light than cells in the log phase. The same relative changes in sensitivity as above have been shown to exist in E. coli B/r when irradiated with x-ray (Stapleton, 1955; Brownell, 1955).

A great deal of the data reported to date has been explained, either directly or indirectly, on the basis of alterations in metabolism. It is fairly well established that pre- and postirradiation modifications of x-ray damage can be produced by conditions which alter metabolism. Two of the more convincing reports that indicate metabolism may be involved in changes of radiosensitivity are the works of Stapleton, Billen, and Hollaender (1952); and Stapleton and Engel (1960).

The former found that a protection against the lethal effects of x-irradiation was obtained when cell suspensions of E. coli B/r were preincubated with pyruvate, formate, succinate, serine, or ethanol. They found too, that protection was not obtained when incubation was carried out at 2 C, suggesting that metabolism is required for the protective effect of these compounds.

The latter observed that the resistance conferred by growth of E. coli in the presence of glucose was directly related to the final pH of the culture in the stationary phase of growth. They concluded that this resistance was not an effect of pH per se, since the pH of the suspending medium during irradiation did not alter the sensitivity of either sensitive or resistant cell populations. Moreover, cells cultured in a glucose medium kept at a constant pH during the growth cycle did not show the radioresistance characteristic of that pH. These cells only became radioresistant if the pH was allowed to decrease as a result of acid production in the course of metabolism of a substrate by the organism.

Radiosensitivity of microorganisms has been found to change during the growth cycle. Likewise, physical alterations which affect the metabolism of the cell alters its radiosensitivity. A study of metabolic changes throughout the growth cycle, and an attempt to determine if these changes correlate with changes in radiosensitivity would be very meaningful. Through this type of study it might be possible to correlate changes in radiosensitivity with the activity or inactivity of certain metabolic processes.

It has been shown previously that the radiosensitivity of Staphylococcus aureus changes during the growth cycle, and that these changes can be directly correlated with the various phases of growth (Howell, 1964). Before it can be determined if alterations in the metabolism can account for

these observed results, it becomes necessary to alter intentionally the metabolism by altering cultural conditions, and noting if this in turn changes the pattern of radiosensitivity in this system.

The first part of this dissertation deals with studies of factors affecting radiation response. The second and third sections deal with changes in the metabolism of the cells under one set of environmental conditions, and a study on the effect of adaptation on x-ray sensitivity. A correlation of the data along with possible mechanisms is presented in the discussion section.

CHAPTER II

MATERIALS AND METHODS

Culture: Staphylococcus aureus obtained from the University of Oklahoma stock culture collection was used in all studies. Stock cultures were maintained on a variety of media depending on the experiment being conducted. All stock cultures were transferred at 24 hr intervals, and incubated at 37 C unless otherwise noted. Three 24 hr transfers were made before each experiment.

Factors Affecting Radiosensitivity

Cell age: Twenty-four hour old cells were harvested from nutrient agar or brain heart infusion agar by washing with 0.85% saline. This saline suspension was used to inoculate a fresh broth medium of the same type to an optical density of 0.17, at 575 mμ, on a Bausch and Lomb Spectronic 20 Colorimeter. Previous experiments have shown that a cell suspension adjusted to this optical density is equivalent to approximately 2.5×10^8 cells/ml. Cultures were incubated at 37 C, and samples were taken from the broth culture at 2 hr intervals, starting at zero time, through 12 hr. Growth of the cells in the broth medium was followed by recording

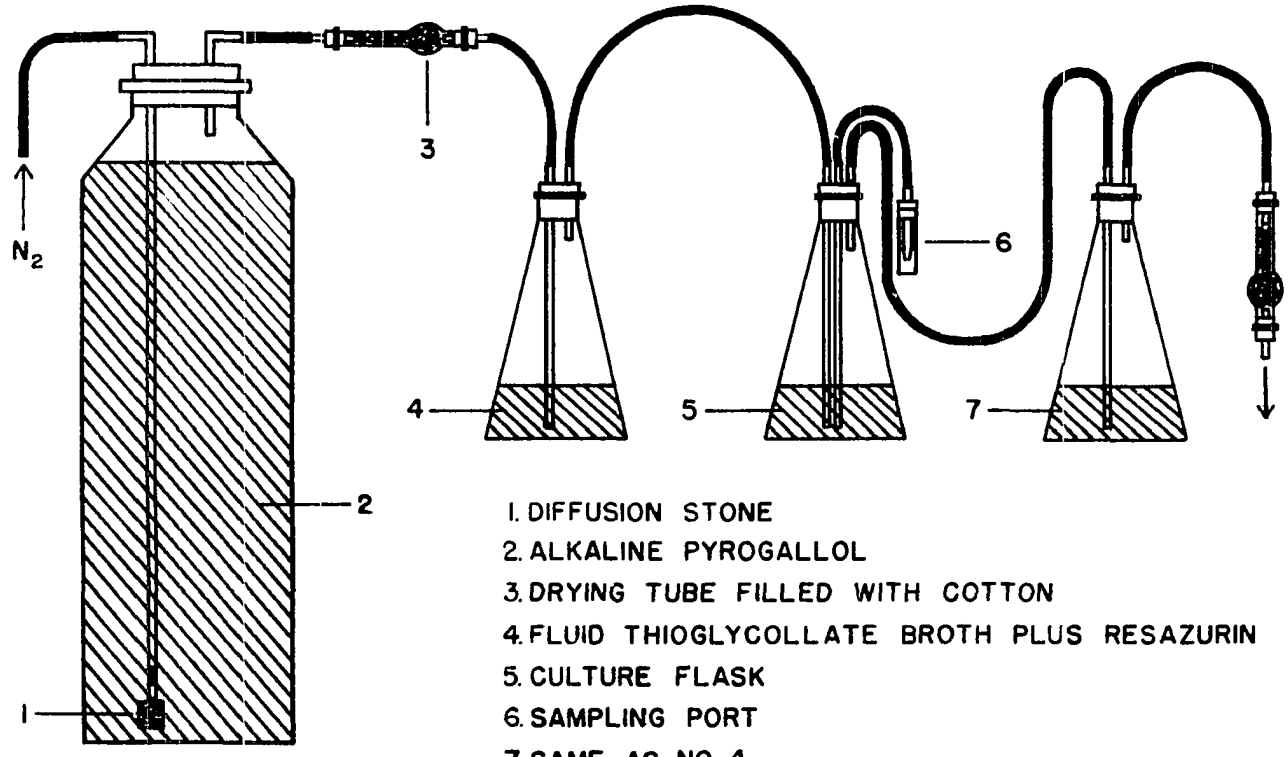
the optical density at 575 mμ.

Irradiation procedures: Samples taken at the various time intervals were exposed to unfiltered x-radiation with a Machlett OEG 60 x-ray tube operating at 70 kv and a dose rate of 300 r/min. Dosimetry was measured using a Victoreen Model R rate meter. Samples were stirred with a magnetic stirrer to insure an even distribution of cells in the sample, and held at approximately 5 C throughout the entire operation. Aliquots were removed before and during x-ray treatment and plated on media of the same type used for preirradiation growth. Where the recovery medium was different from preirradiation growth medium it will be noted. After an incubation period of 24-48 hr at 37 C, percent survival was calculated from plate counts of irradiated and nonirradiated samples.

Effect of anaerobic growth conditions on changes of radiosensitivity with cell age: Cells previously grown aerobically were inoculated into freshly autoclaved and cooled brain heart infusion broth and grown anaerobically. Three successive 24 hr transfers were made before the experiments to allow for cell adaptation to growth under anaerobic conditions.

The apparatus shown in Figure 1 was used to obtain anaerobic conditions during growth. Nitrogen gas was passed through alkaline pyrogallol. After passing through this solution the nitrogen passed through freshly autoclaved fluid thioglycollate broth (Difco) containing resazurin as an

Figure 1. Apparatus used to maintain anaerobic growth conditions.



1. DIFFUSION STONE
2. ALKALINE PYROGALLOL
3. DRYING TUBE FILLED WITH COTTON
4. FLUID THIOGLYCOLLATE BROTH PLUS RESAZURIN
5. CULTURE FLASK
6. SAMPLING PORT
7. SAME AS NO. 4

oxygen indicator. In the presence of oxygen this medium will turn pink. The oxygen free nitrogen was bubbled through the culture flask and then through another flask of fluid thioglycollate broth plus resazurin.

A slight positive pressure was maintained in the culture flask which facilitated the removal of samples, and prevented contamination of the culture flask with oxygen.

Irradiation was conducted under a nitrogen atmosphere using oxygen free nitrogen. When recovery was anaerobic, cultures were grown in Prickett tubes containing brain heart infusion agar plus 0.1% sodium thioglycollate.

Irradiation and calculation of percent survival procedures were those previously described.

Effects of pre- and postirradiation growth medium on radiosensitivity: Twenty-four hour old cells of S. aureus were harvested from nutrient agar, brain heart infusion agar, or a solidified synthetic medium by procedures previously described. The composition of the synthetic medium is given in Table I (Gale, 1947). Modifications made in this medium are listed as such. It was found that this modified medium gave faster growth and higher total cell numbers. Cell suspensions were inoculated into a broth medium of the same type to give an optical density of 0.17, at 575 m , and incubated at 37 C.

Experiments were conducted on the comparative radiosensitivity of late log phase cells grown in nutrient broth

TABLE I

Synthetic Medium

A.	Amino Acids	μM each L-isomer/ml
1.	L-arginine-HCL	0.5
2.	L-cysteine	0.5
3.	L-histidine-HCL	0.5
4.	L-lysine-HCL	0.5
5.	DL-isoleucine	0.5
6.	L-leucine	0.5
7.	DL-methionine	0.5
8.	L-phenylalanine	0.5
9.	L-proline	0.5
10.	DL-serine	0.5
11.	DL-threonine	0.5
12.	DL-tryptophane	0.5
13.	L-tyrosine	0.5
14.	L-valine	0.5
15.	L-alanine	1.25
16.	L-aspartic	1.25
17.	L-glutamic	1.25
18.	glycine	1.25

B.	Vitamins	μg/ml
1.	thiamine	2.0
2.	nicotinic acid	2.0
3.	pyridoxine	2.0

C.	Additional	g/ml
1.	glucose	0.1

Modifications:

A.	Vitamins	μg/ml
1.	biotin	2.0
2.	choline	2.0
3.	folic acid	2.0
4.	pantothenate	2.0
5.	riboflavin	2.0
6.	PABA	2.0

B.	Purine and Pyrimidine Bases	μg/ml
1.	adenine	20
2.	guanine	20
3.	thymine	20
4.	cytosine	20
5.	uracil	20

C.	Salts	mg/liter
1.	$FeSO_4 \cdot H_2O$	1.0
2.	$CuSO_4 \cdot 5H_2O$	1.0
3.	$ZnSO_4 \cdot 7H_2O$	1.0
4.	$MnSO_4 \cdot 4H_2O$	1.0
5.	$MgSO_4$ (anhy)	1.0

D.	Additional	g/liter
1.	NaCl	5.0
2.	Dipotassium phosphate	5.8

and plated on nutrient agar; grown in brain heart infusion broth and plated on nutrient agar; grown in synthetic broth and plated on synthetic agar; grown in synthetic broth and plated on brain heart infusion agar; grown in brain heart infusion broth and plated on synthetic agar; and grown in brain heart infusion broth and plated on brain heart infusion agar.

Irradiation and calculation of percent survival procedures were those previously described.

Preparation of synthetic medium: Amino acids, vitamins, glucose, salts, purine and pyrimidine bases, and sodium chloride-dipotassium phosphate were all prepared in separate 10X solutions. All solutions except vitamins and glucose were sterilized by autoclaving. Vitamin and glucose solutions were sterilized by filtration (Millipore Filter Corp., Type HA 0.45μ).

The synthetic medium was constructed by adding, aseptically, aliquots of the various solutions to a sufficient amount of sterile distilled water to give a final concentration of each component as shown in Table 1.

When solid media were used, agar-agar was added to distilled water, autoclaved, and appropriate amounts of each 10X solution added after the agar solution was cooled to 50 C in a water bath.

Effects of temperature during irradiation on radiosensitivity: Six hour old cells were harvested from brain

heart infusion agar and diluted in 0.85% saline to give approximately 2 x 10^4 cells/ml. The effect of temperature during irradiation on radiosensitivity was determined by maintaining cell suspensions at predetermined temperatures and exposing to x-radiation. Percent survival was calculated from plate counts of irradiated and nonirradiated samples grown on brain heart infusion agar.

Metabolic studies during the life cycle

Fermentation studies: Twenty-four hour old cells were washed from brain heart infusion agar with 0.85% saline and used to inoculate 6 liters of fresh brain heart infusion broth. The broth medium suspension was standardized to an optical density of 0.17, at 575 mμ, and at 0, 2, 4, 6, 8, 10, 12, and 24 hr, samples were removed. Each sample was centrifuged at 10,000 X G, at 4 C, for 10 min. The growth medium was decanted, sterilized by filtration (Millipore Filter Corp., HA 0,45μ), and stored at -20 C.

The following determinations were made on all samples:

1. Total carbohydrate
2. Organic matter
3. Amino nitrogen
4. Protein
5. Free amino acids
6. Glycerol
7. Fermentation acids
8. Simple aliphatic alcohols
9. Diacetyl
10. Acetoin
11. Acetone
12. Indol

*13. pH
*14. Relative oxidation-reduction potential

*Determinations not made on above samples but in separate experiments.

Methods of analysis used in the above determinations:

Total carbohydrate, as glucose, was determined by the method of Morris (1948). Results obtained by this procedure indicated that other carbohydrates were present since the values were slightly higher than could be accounted for by glucose alone. To determine the presence of other carbohydrates, chromatographic techniques were employed. Samples of uninoculated brain heart infusion broth were spotted on Whatman No. 3MM filter paper, and one dimensional descending development carried out in n-butanol-pyridine-water-glacial acetic acid (60:40:30:5) (Primosigh et al., 1961). Sugars were detected by spraying with aniline acid phthlate reagent (2.0 ml aniline, 3.3 g phthalic acid, 100 ml water saturated with n-butanol), and heated 10 min at 100 C (Becker et al., 1965).

Organic matter was determined colorimetrically by the method of Johnson (1949).

Amino nitrogen determinations were made using the ninhydrin method of Cocking and Yemm (1954).

The protein content of all samples was determined by use of the Folin-Ciocalteau test (Lowry et al., 1951).

Free amino acids were determined by spotting aliquots on Whatman No. 3MM filter paper (46 cm x 46 cm), and subjecting to two dimensional descending chromatography with

secondary-butanol-formic acid-water (70:10:20) as the first solvent followed by aqueous phenol (80% w/v) containing 0.3% (v/v) ammonia (sp gr 0.89) in the second dimension (Hancock, 1958). After drying, amino acids were detected by spraying with 0.5% (w/v) ninhydrin in acetone, and developed by heating at 100 C for 10 min. Components present were identified by comparison with the position of authentic amino acid markers.

Glycerol determinations were made using the method of Lambert and Neish (1950).

Fermentation acids were obtained by first clearing the fermentation samples using the zinc sulfate method of Somogyi (1930). The resulting cleared solution was acidified to pH 2 with 1 N hydrochloric acid and ether extracted. A known quantity of preboiled water was added to the ether phase, and the ether evaporated at 50 C. All samples were stored in a desiccator for 24 hr prior to analysis.

To each sample was added 0.5 ml of 0.21 N hydrochloric acid, and the fermentation acids determined by partition chromatography on a silica-water column using the method of Neish (1949). Acids analyzed for were acetic and lactic. The amount of each acid was determined by titration with 0.01 N sodium hydroxide to a phenol red end point.

Lactic acid was also determined colorimetrically by the method of Barker and Summerson (1941) as modified by Markus (1950).

Alcohols analyzed for included n-butanol, isopropanol,

and ethanol. Separation was carried out by use of partition chromatography on a Celite-water column using carbon tetrachloride and chloroform as developing solvents. Fractions were collected, and each alcohol measured by dichromate oxidation followed by measurement of dichromate consumed (Neish, 1951).

Diacetyl was determined colorimetrically by the method of White et al.,(1946).

Acetoin plus diacetyl was determined colorimetrically by the method of Westerfield (1945) as modified by Eggletor et al.,(1943).

Acetone was determined by the method of Snell and Snell (1937).

The presence of indol was tested for by the use of Kovac's reagent (n-amyl alcohol, 75 ml; conc. hydrochloric acid, 25 ml; para-dimethylamine-benzaldehyde, 5 g).

To determine pH or oxidation-reduction changes during growth of a culture, a 250 ml flask of brain heart infusion broth was inoculated to an optical density of 0.17, at 575 mμ, with a 24 hr old culture of S. aureus. The flask was fitted with a rubber stopper so constructed that a Beckmann combination electrode (No. 39812 for determination of pH) or a Beckmann combination platinum electrode (No. 39186 for determination of oxidation-reduction potential) could be inserted through it into the culture fluid. Changes were recorded using a Beckmann Zeromatic pH meter in connection with a

Bausch and Lomb Model V.M.O.-5 Recorder.

Optical density measurements for all colorimetric reactions were carried out using a Model 2000 Gilford Multiple Sample Absorbance Recorder connected to the monochromator of a Beckmann Model DUR Recording Spectrophotometer.

Cellular metabolic studies: Metabolic studies were conducted on cells grown on brain heart infusion broth. Cells of S. aureus were maintained on brain heart infusion agar, and incubated at 37 C for 24 hr. These cells were harvested by washing the cells from the agar with saline. Saline suspended cells were used to inoculate brain heart infusion broth to an optical density of 0.17; at 575 mμ. At 2 hr intervals through 12 hr cells were harvested by centrifugation at 4 C, at 10,000 X G. All cell suspensions were washed four times in 0.1 M phosphate buffer, pH 7.

Oxygen uptake and carbon dioxide production were determined using the Manometric techniques described in Umbreit et al., (1964). The flask contained: 0.2 ml of 20% potassium hydroxide; 0.2 ml of 0.1 M substrate; and 2.7 ml of cell suspension. The gas phase in all experiments was air except in experiments measuring carbon dioxide production by anaerobically grown cells where the gas phase was oxygen free nitrogen. Results are reported as microliters of oxygen uptake or carbon dioxide production per mg dry weight per hour.

Whole cells, prepared as previously described, were used in all experiments.

Nuclear stains were made using the procedure of Chance (1952).

Relative amounts of nucleic acids were determined using the hydrolysis procedure of Morse and Carter (1949), and the colorimetric procedures of Kerr and Seraidarian (1945) and Sevag et al., (1940).

Adenosine triphosphate (ATP) was determined quantitatively in cells harvested at 0, 2, 5, 8, and 10 hr after inoculation into brain heart infusion broth. The enzyme luciferase was used in all determinations. Procedures used were those described by Billen et al., (1953).

Light intensity measurements were made using the photomultiplier tube in the monochromator of a Beckmann Model DUR Recording Spectrophotometer connected to a Model 2000 Gilford Multiple Sample Absorbance Recorder.

CHAPTER III

RESULTS

Figure 2 shows the typical radiosensitivity curve for Staphylococcus aureus grown in brain heart infusion broth (Howell, 1964). Controls and irradiated samples were plated on brain heart infusion agar. Direct correlation between the changes in radiosensitivity and changes in the growth cycle can be seen. During the lag phase, cells become more resistant to the lethal effects of x-ray. As cell division commences the cells become more sensitive, and this pattern is seen to continue through the log phase of growth. In the late log phase, as cell division starts to decline, resistance again increases. At about 8 hr, or as the cells start entering the stationary phase of growth, radiosensitivity increases.

To determine if this phenomenon could be demonstrated in cells grown on different media, the same type of experiment was conducted with cells grown in nutrient broth. In this experiment controls and irradiated samples were plated on nutrient agar. The results are shown in Figure 3.

The changing of the growth medium did not alter the

Figure 2. Changes in radiosensitivity as a function of cell age.

Growth medium was brain heart infusion broth. Controls and irradiated samples were plated on brain heart infusion agar. (1) Changes in optical density corresponding to growth. (2) Sensitivity changes as a function of cell age following a dose of 4500 r.

All cultures were grown aerobically.

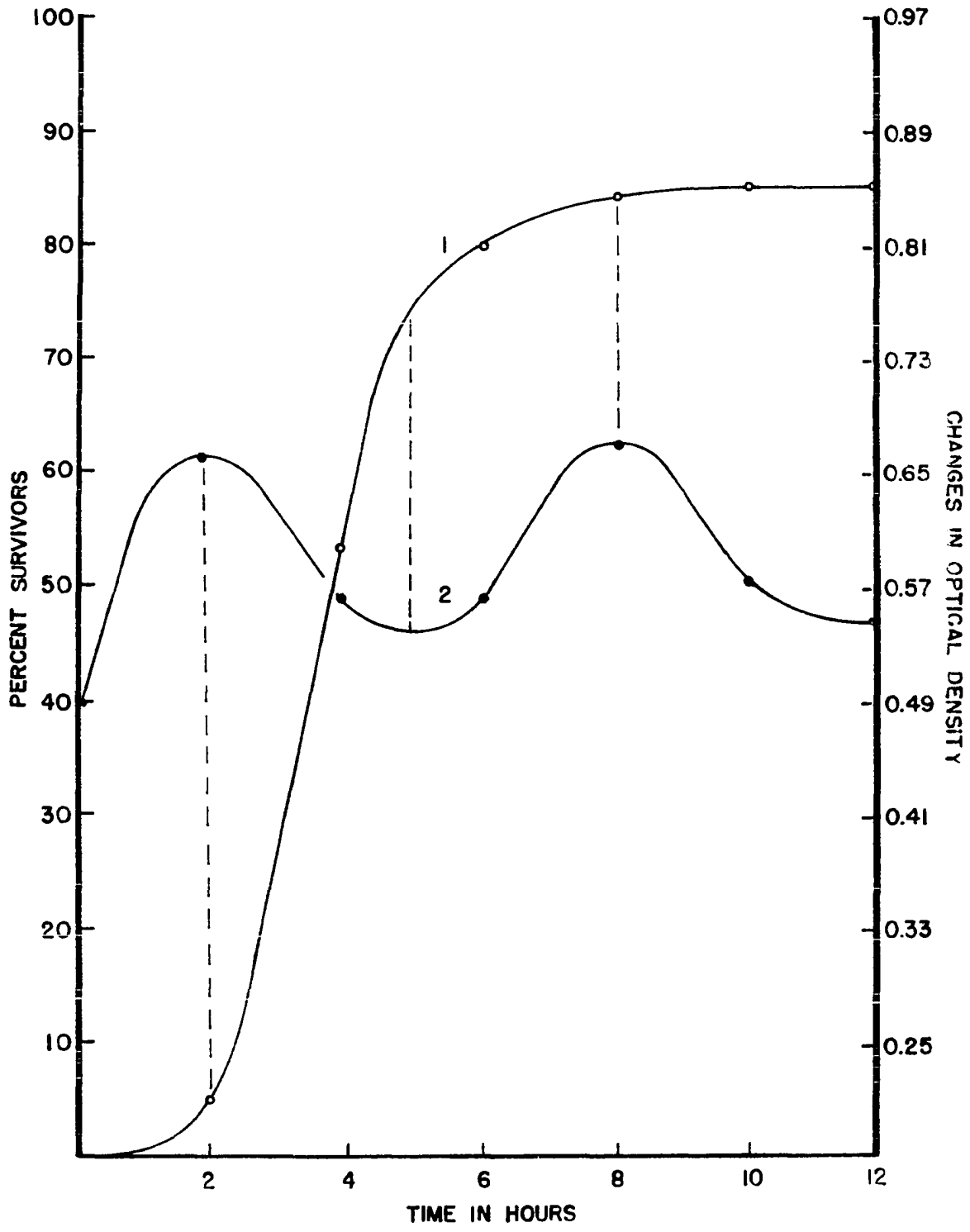
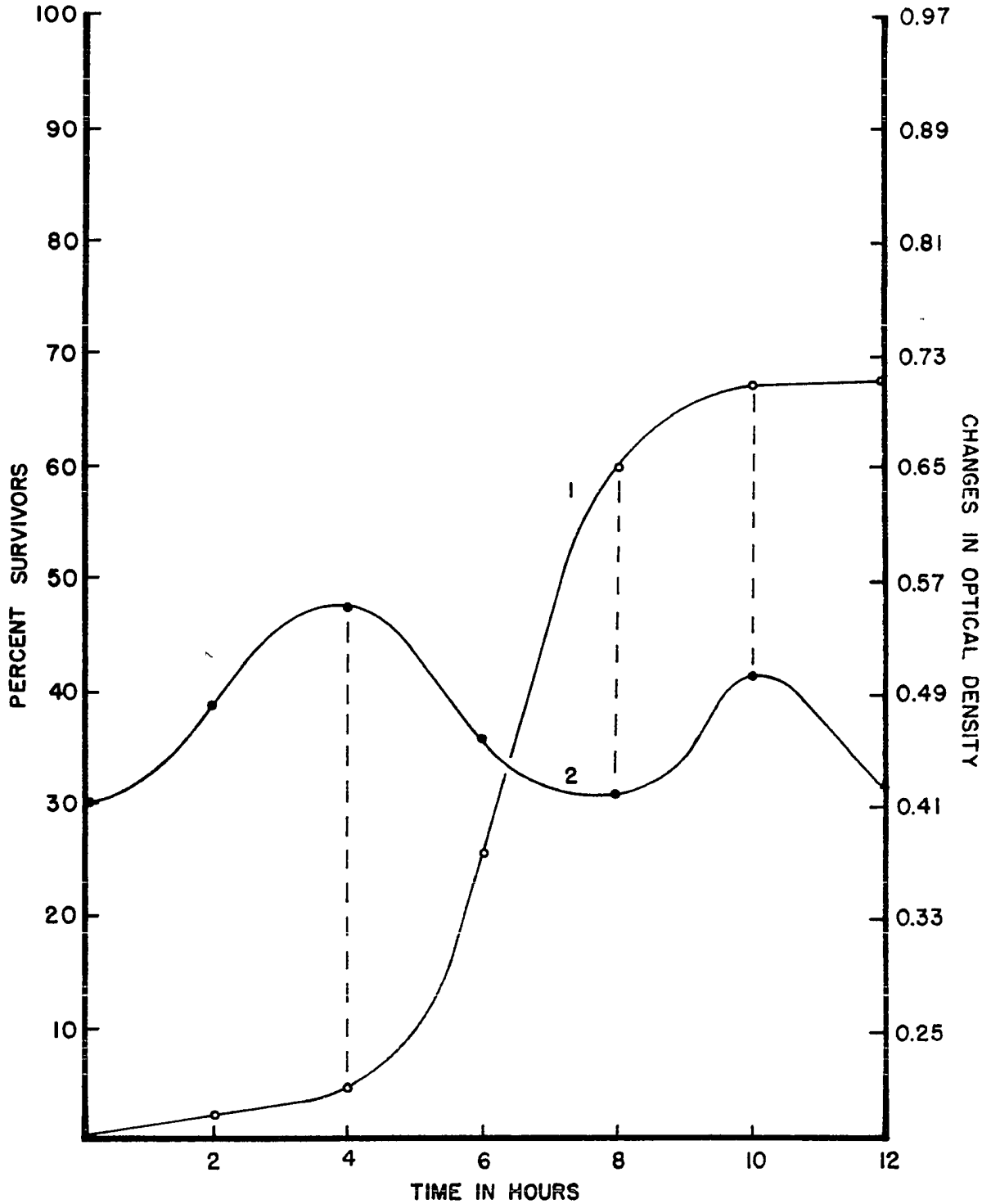

100
90
80
70
60
50
40
30
20
10
0.97
0.89
0.81
0.73
0.65
0.57
0.49
0.41
0.33
0.25
1
2
PERCENT SURVIVORS
CHANGES IN OPTICAL DENSITY
2
4
6
8
10
12
TIME IN HOURS

Figure 3. Changes in radiosensitivity as a function of cell age.

Growth medium was nutrient broth. Controls and irradiated samples were plated on nutrient agar. (1) Changes in optical density corresponding to growth. (2) Sensitivity changes as a function of cell age following a dose of 4500 r.

All cultures were grown aerobically.


100
90
80
70
60
50
40
30
20
10
PERCENT SURVIVORS
0.97
0.89
0.81
0.73
0.65
0.57
0.49
0.41
0.33
0.25
CHANGES IN OPTICAL DENSITY
1
2
2
4
6
8
10
12
TIME IN HOURS

place of appearance of the various resistnace maxima and minima indicating that these changes are independent of growth conditions, but dependent upon phase of growth.

The total sensitivity is dependent upon growth conditions. Cells grown in nutrient broth were less resistant to the lethal effects of x-ray than cells grown in brain heart infusion.

When growth was in nutrient broth, lag phase cells were more resistant than were stationary phase cells. In brain heart very little difference in the sensitivity of these two cell populations was observed. The place of appearance of increased resistance during growth (late lag and early stationary phases) is independent of growth conditions, but the total resistance obtained at these times is dependent upon growth conditions. This fact is further demonstrated in Figure 4.

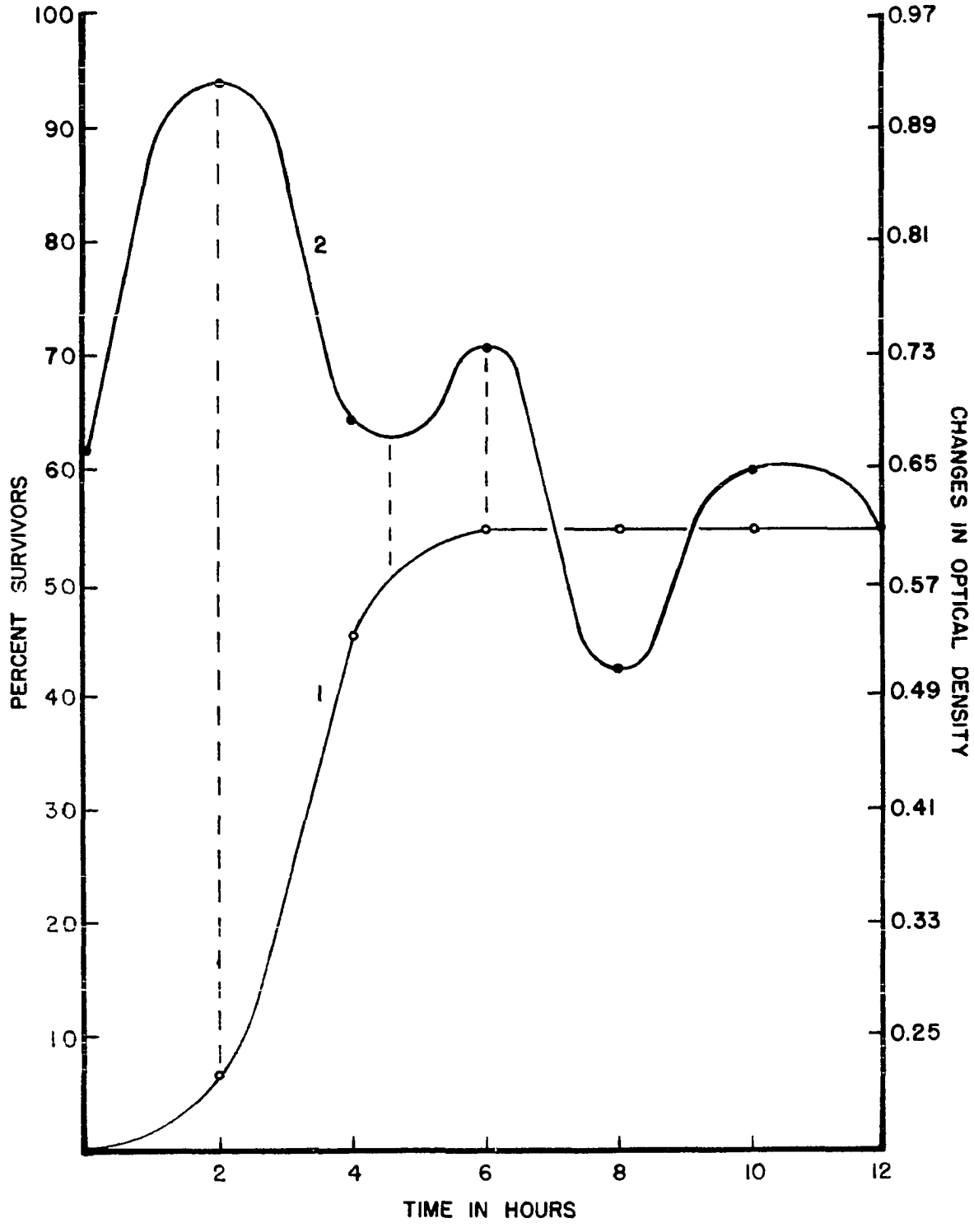
Previously anaerobically grown cells were inoculated into fresh brain heart infusion broth and grown anaerobically. Samples were taken from this culture at various time intervals, and exposed to 4500 r of x-radiation. Irradiated and nonirradiated samples were grown anaerobically.

It is noted that various maxima and minima in sensitivity occur. These again can be directly correlated to the different phases of growth. The degree to which each is affected is dependent upon environmental conditions. Late lag phase cells in this case show a much higher resistance

Figure 4. Changes in radiosensitivity as a function of cell age.

Growth medium was brain heart infusion broth. Controls and irradiated samples were plated on brain heart infusion agar. (1) Changes in optical density corresponding to growth. (2) Sensitivity changes as a function of cell age following a dose of 4500 r.

All cultures were grown anaerobically.


100
90
80
70
60
50
40
30
20
10
PERCENT SURVIVORS
0.97
0.89
0.81
0.73
0.65
0.57
0.49
0.41
0.33
0.25
CHANGES IN OPTICAL DENSITY
2
1
2
4
6
8
10
12
TIME IN HOURS

than do late log phase cells.

The total sensitivity of the anaerobically grown culture is much less than that observed with either of the two previous aerobic systems. This fact is not surprising, since this same phenomenon has been demonstrated in many other organisms (Zelle and Hollaender, 1955; Kimball, 1957; De Serres, 1961).

These gross changes can not be attributed to total growth of the population. Total numbers of cells, in anaerobically grown culture, were less than in either of the aerobically grown systems, yet resistance was greater. Total cell numbers in brain heart infusion broth were higher than those observed in nutrient broth, and total resistance was highest in the former.

The pattern of sensitivity changes which occur during the growth cycle are independent of different homogeneous growth conditions (pre- and postirradiation growth conditions the same), but the degree to which each maximum and minimum changes is directly dependent upon these conditions. Because of this it would appear that there must exist some metabolic function common to all cultures in the lag phase, another common to all cultures in the log phase, and another common to all cultures in the stationary phase which is independent of environmental conditions.

To study changes in metabolism and determine if the changes observed were associated with changes in x-ray

sensitivity, one of the three environmental systems just described was chosen. The following studies were conducted on aerobically grown cells using brain heart infusion broth as the growth medium. Figure 2 is a reference of changes in radiosensitivity during growth under these conditions.

Several investigators have suggested that multicellular or multinuclear states could account for the differences observed between sensitive and resistant cell populations (Birge and Tobias, 1954; Howard, Flanders and Alper, 1957; Sargent, 1958). Stapleton and Engel (1960) could not demonstrate this factor as being the cause of this effect, and their results indicated a metabolic cause.

Microscopic observations of 24 hr old cells (0 hr cells in Figure 2), and 2 hr old cells stained by the method of Chance (1952), demonstrated that many of the 2 hr cells were multinucleate. These cells are also about 2 to 3 times larger than 24 hr old cells. The possibility that increased intracellular deoxyribonucleic acid (DNA) content might account for increases in survival at this time was studied.

Deoxyribonucleic acid analysis on a viable cell basis indicated an increased DNA content. Analysis on a dry weight basis per viable cell indicated that the ratio of DNA content of resistant cells to that of sensitive cells was not different enough to account for the increased resistance (ratio = 1.15:1). The ratio of ribonucleic acid (RNA) content was slightly larger than that observed for DNA (ratio = 1.3:1).

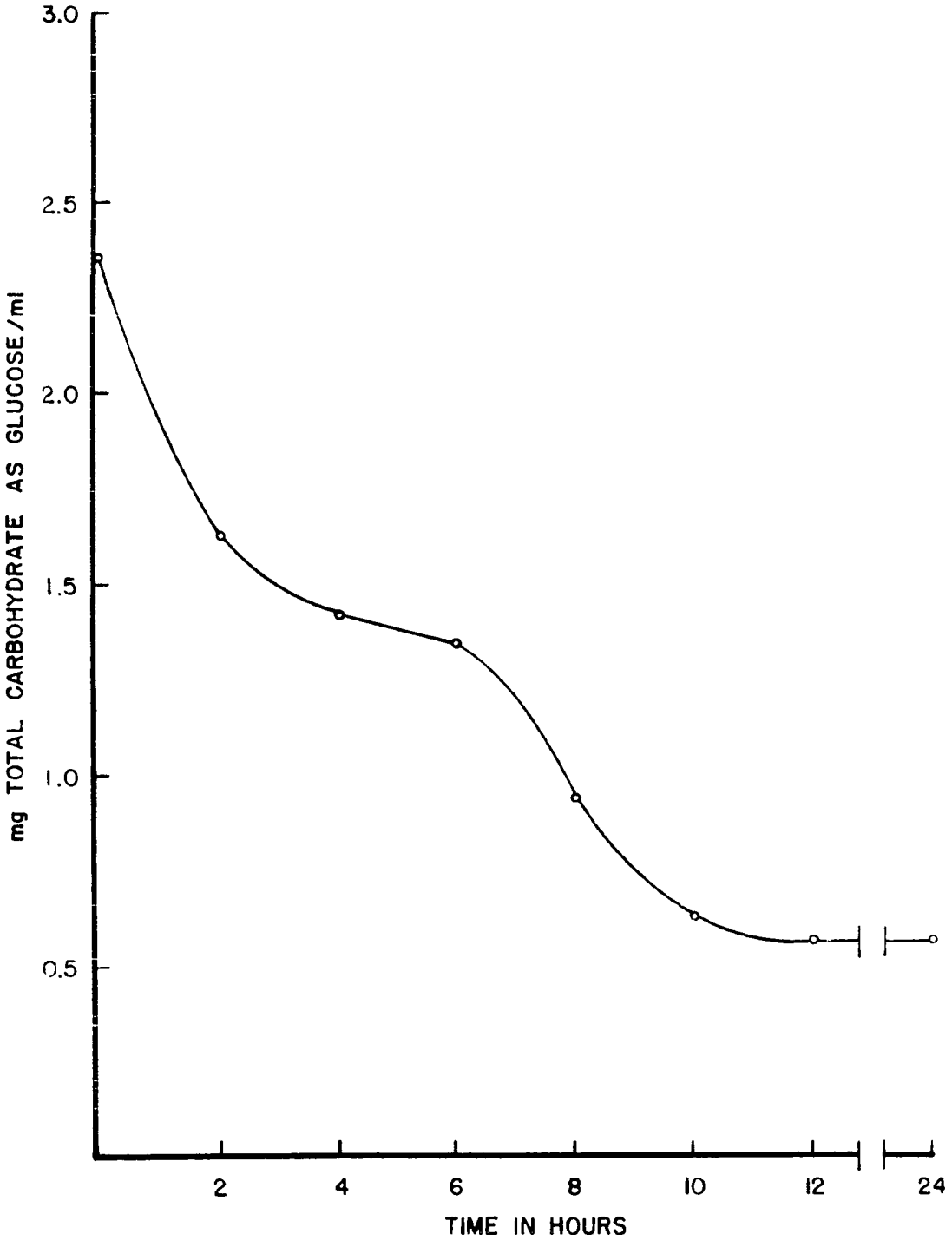
Ratios of the resistant late lag phase cells to the sensitive middle log phase cells (4 hr old cells in Figure 2) showed that DNA and RNA ratios were approximately 1:1 (DNA ratio = 1.01:1 and RNA ratio = 1.1:1). It is evident, therefore, that the changes in radiosensitivity can not be attributed to the increase or decrease of these components.

Increased size of the 2 hr old cells correlated with increased resistance, since cell size at 4 hr was much smaller. However, this does not correlate with the increased resistance at 8 hr since cell size continually becomes smaller after the first 2 hr (Savage, 1966).

In this initial study it became impossible to study the complete cellular metabolism throughout the growth cycle. Since analysis of the growth medium reflects, indirectly, the metabolism of the cells, this system was chosen. At various time intervals samples of the growth medium were taken, and analyzed for the appearance or disappearance of certain components by procedures previously described.

Figure 5 shows results of the analysis of total carbohydrate as glucose at various time intervals after inoculation. During the first 2 hr of growth total carbohydrate decreased rapidly. From 2 hr to 4 hr utilization leveled off, and this leveling off continued until 6 hr. Between 6 hr and 8 hr the rate of disappearance increased, and leveled off again between 10 hr and 12 hr. The total carbohydrate level in 12 hr spent medium was only slightly

Figure 5. Total carbohydrate, as glucose, in brain heart infusion broth at various times after inoculation.


3.0
2.5
2.0
1.5
1.0
0.5
mg TOTAL CARBOHYDRATE AS GLUCOSE/ml
2
4
6
8
10
12
24
TIME IN HOURS

higher than that found in 24 hr spent medium.

A comparison of Figure 2 with Figure 5 shows that when the rate of carbohydrate disappearance was the most rapid, from 0 hr to 2 hr, and 6 hr to 8 hr, resistance to x-ray increased. This is better demonstrated in Figure 6 where total carbohydrate disappearance is plotted per unit time. The similarity between data shown in Figure 6, and changes in radiosensitivity shown in Figure 2 is striking.

Brain heart infusion contains 2.0 mg of glucose per ml. Analysis of total carbohydrate indicated the presence of about 2.3 mg of total carbohydrate per ml in uninoculated medium. To determine if carbohydrates other than glucose existed in this medium, chromatographic analysis of the medium was conducted. No sugars other than glucose could be detected. No substances are known to react with anthrone to give a color reaction except carbohydrates. Aldonic acids and sugar alcohols do not react. Since the growth medium was not hydrolyzed prior to chromatographic analysis, it is possible that this 0.3 mg/ml could be accounted for as bound sugar, but a more likely answer is that it represents experimental error.

Figure 7 shows results from the colorimetric determination of formaldehyde formed on periodate oxidation (glycerol). One mole of glycerol forms 2 moles of formaldehyde when oxidized by periodic acid. This determination is corrected for total carbohydrate present as glucose. At

Figure 6. Rate of utilization of total carbohydrate, as glucose, per unit time.

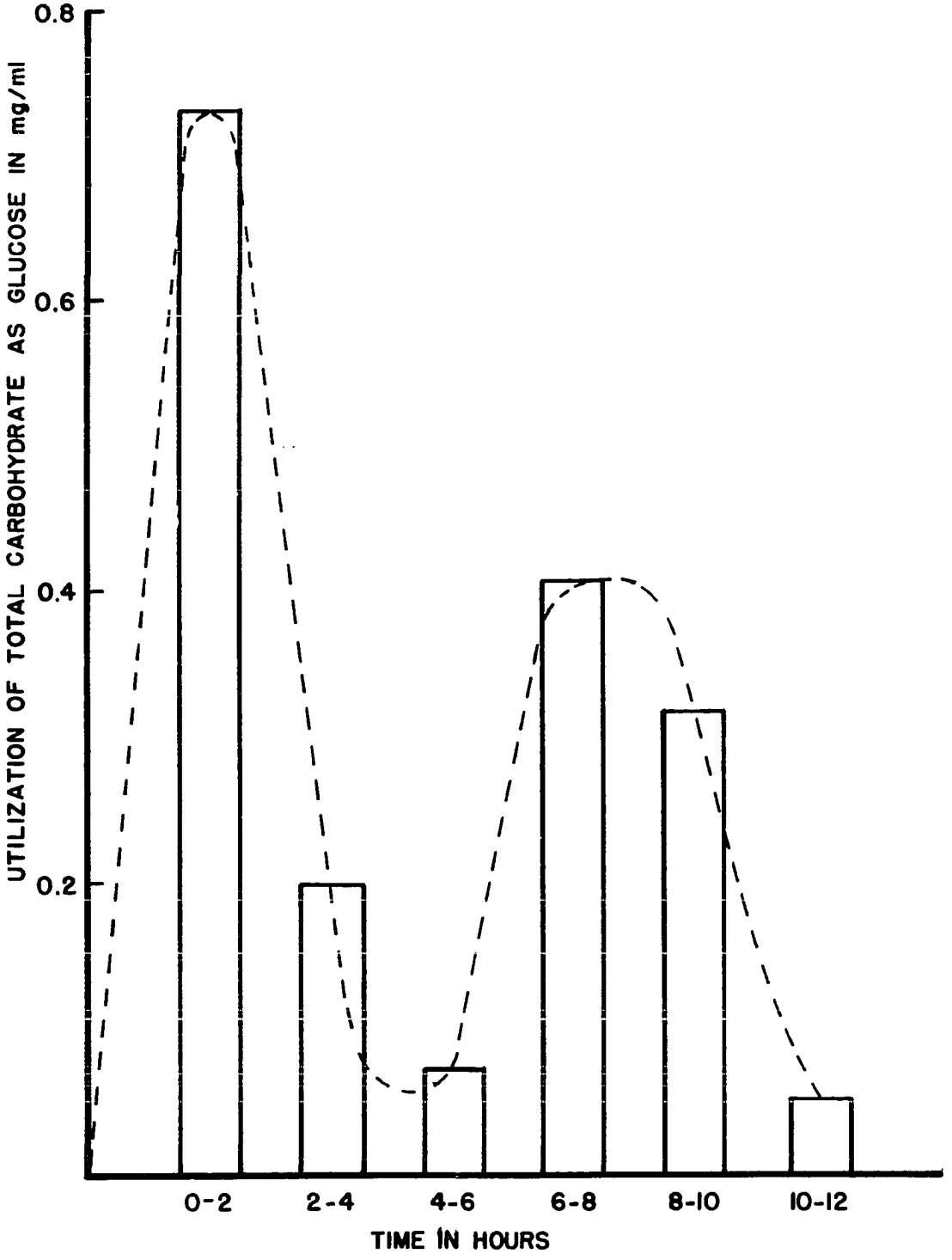
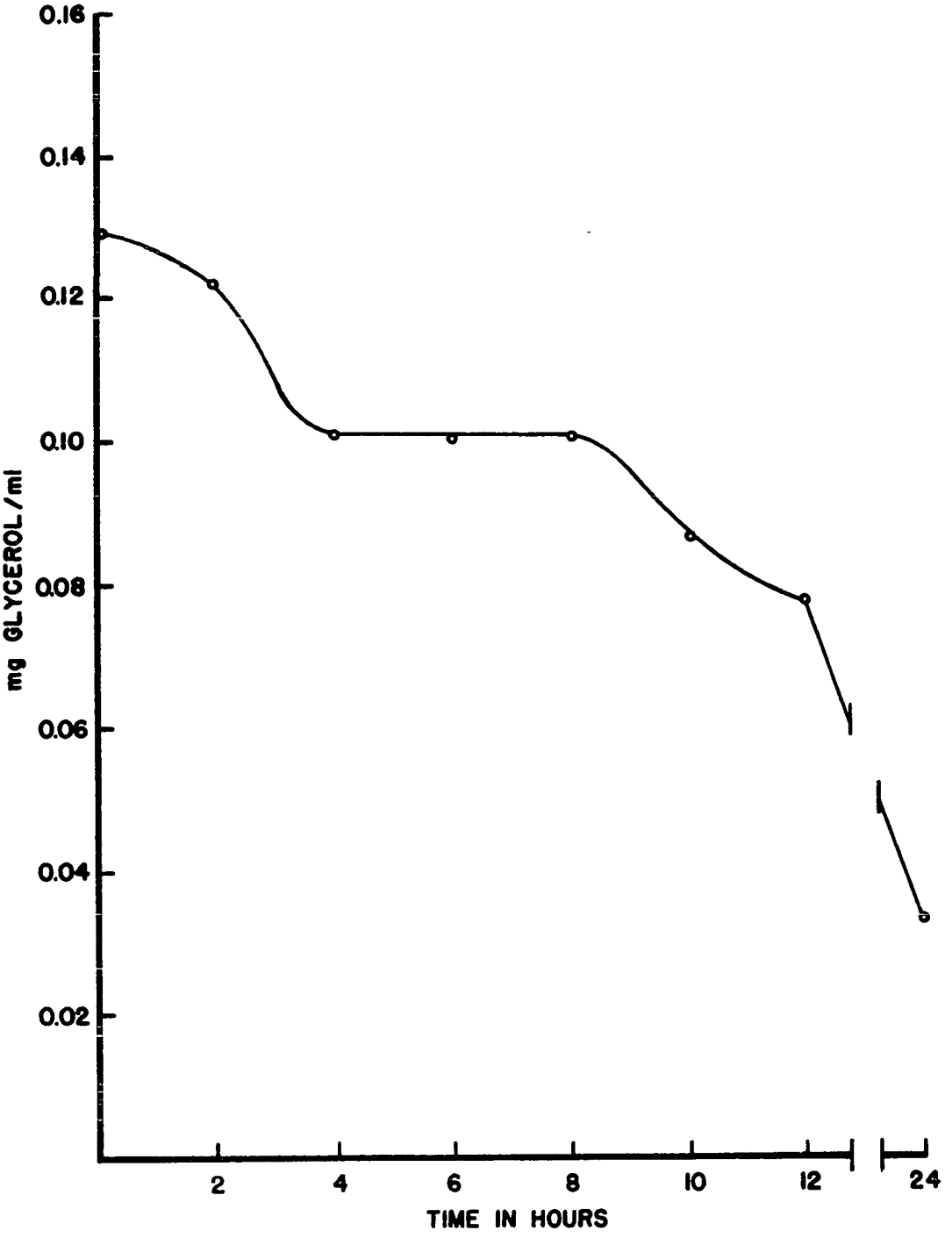

0.8
0.6
0.4
0.2
UTILIZATION OF TOTAL CARBOHYDRATE AS GLUCOSE IN mg/ml
0-2
2-4
4-6
6-8
8-10
10-12
TIME IN HOURS

Figure 7. Glycerol present in brain heart infusion broth at various times after inoculation.


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
mg GLYCEROL/ml
2
4
6
8
10
12
24
TIME IN HOURS

no time during the growth of this organism was glycerol produced.

Glycerol disappeared from the growth medium indicating that it is being utilized as a carbon source. Essentially the same pattern of disappearance was observed here that was observed for glucose disappearance in Figure 5. Also, a correlation between glycerol disappearance and changes in x-ray sensitivity can be made.

Figure 8 shows results from the colorimetric determinations of lactic acid. The level of lactic acid increased until 8 hr. From 8 hr to about 11 hr after inoculation the level decreased, and from 11 hr to 24 hr it again increased. After 24 hr of growth the total lactic acid content was higher than at any other time.

Figure 9 shows the same data plotted as mg of lactic acid per ml produced or utilized per unit time. Production increased during the first 4 hr of growth after which time a decrease was observed. Between 6 hr and 11 hr lactic acid was utilized. Accumulation again appeared at 12 hr.

Chromatographic analysis of acetic and lactic acids indicated that these two acids were produced and utilized at the same times. Lactic acid was produced in much larger quantities than acetic.

At no time during the growth cycle could the presence of diacetyl, acetoin, acetone, indol or alcohols be detected in spent medium. According to the 7th edition of Bergey's

Figure 8. Lactic acid present in brain heart infusion broth at various times after inoculation.

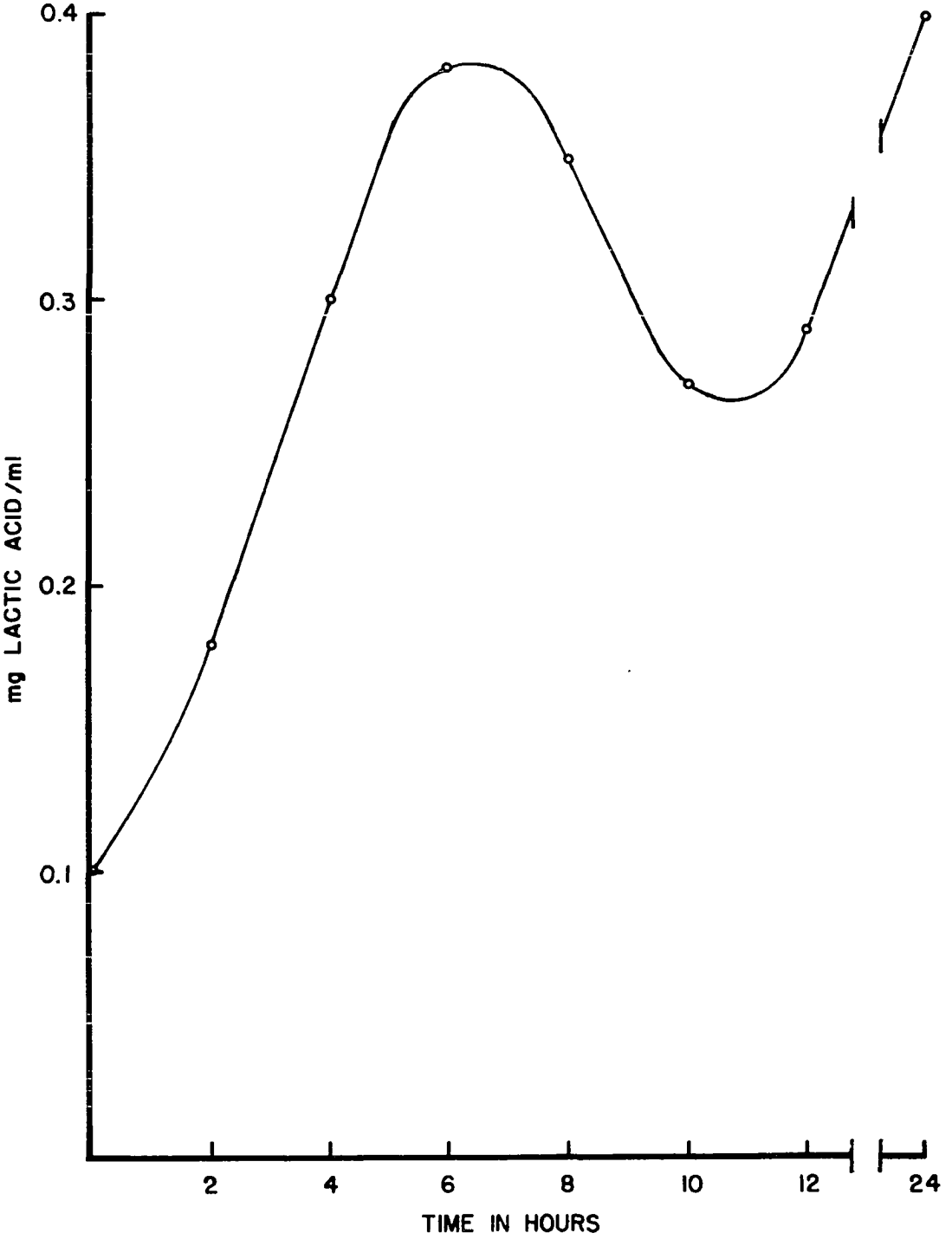
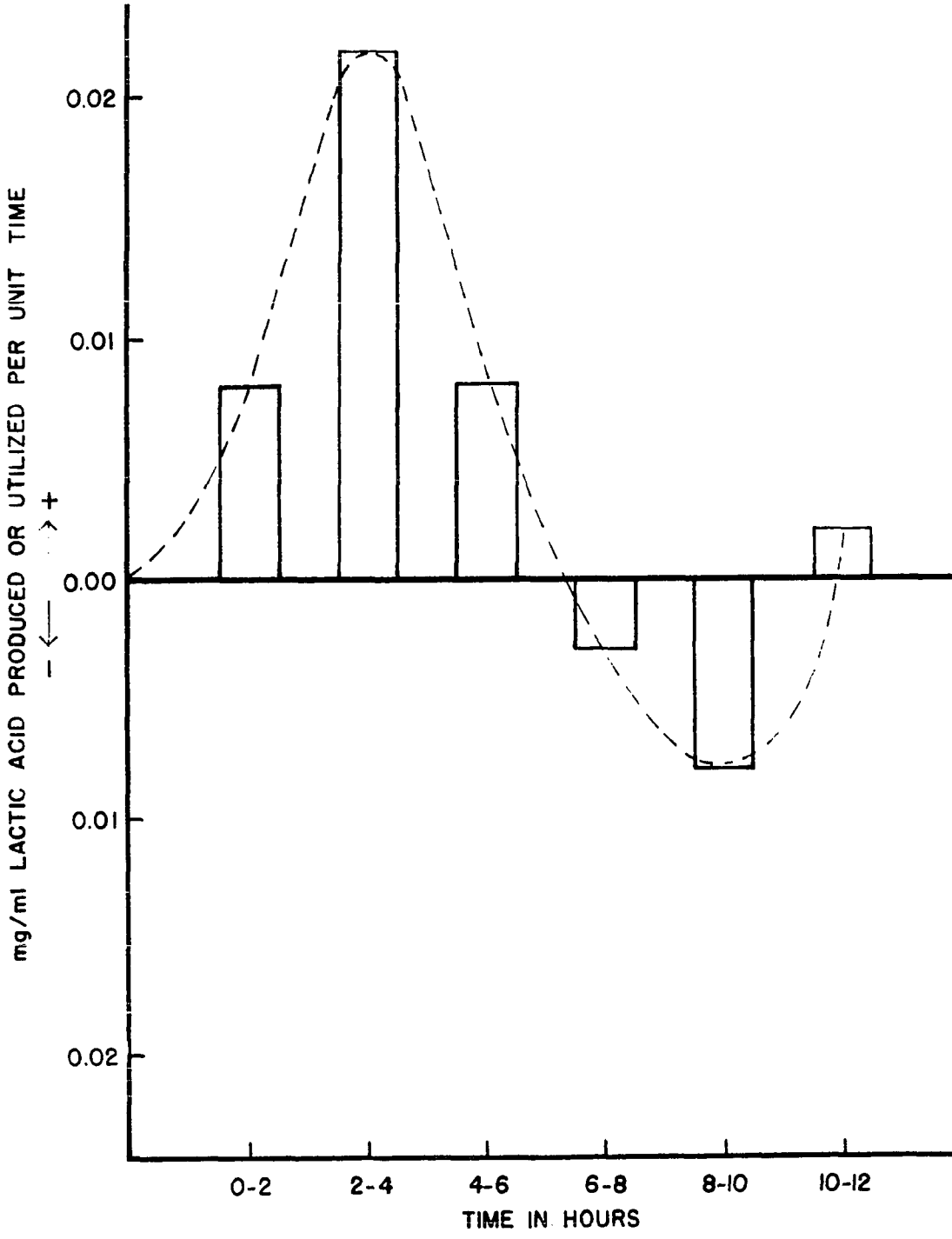

0.4
0.3
0.2
0.1
mg LACTIC ACID/ml
2
4
6
8
10
12
24
TIME IN HOURS

Figure 9. Lactic acid produced or utilized per unit time after inoculation.


0.02
0.01
0.00
0.01
0.02
mg/ml LACTIC ACID PRODUCED OR UTILIZED PER UNIT TIME
− ← → +
0-2
2-4
4-6
6-8
8-10
10-12
TIME IN HOURS

Manual of Determinative Bacteriology (1957), S. aureus produces acetoin and diacetyl in glucose peptone broth. Neither of these compounds could be detected after 24 hr of growth in brain heart infusion.

Figure 10 shows results obtained for mg protein per ml of spent medium. Protein disappearance is essentially confined to the first 4 hr of growth, with the greatest disappearance occuring during the first 2 hr. This time period corresponds to the lag phase of growth (Figure 2).

Amino nitrogen decreased in spent medium up to 4 hr after inoculation (Figure 11). After 4 hr the quantity of amino nitrogen increased. No corresponding drop in protein was detected. After 8 hr the amino nitrogen again decreased.

Ninhydrin is much more sensitive for detection of free amino acids than proteins; whereas, the reverse is true with Folin reagent (Lowry et al., 1951). Therefore, a release of free amino acids into the spent medium would account for the results observed in Figure 11.

Chromatographic analysis of spent medium indicated that this was the case (Table 2). Relative amounts of aspartic, asparagine, alanine, glutamic, leucine, phenylalanine, serine, tyrosine, threonine, valine, and an unknown component all decreased during the first 2 hr of growth. At 8 hr part of these reappeared in quantities equal to or greater than that observed in uninoculated medium. Also, at this time, tryptophane and a compound tentatively identified

Figure 10. Disappearance of protein from brain heart infusion as a function of time after inoculation.

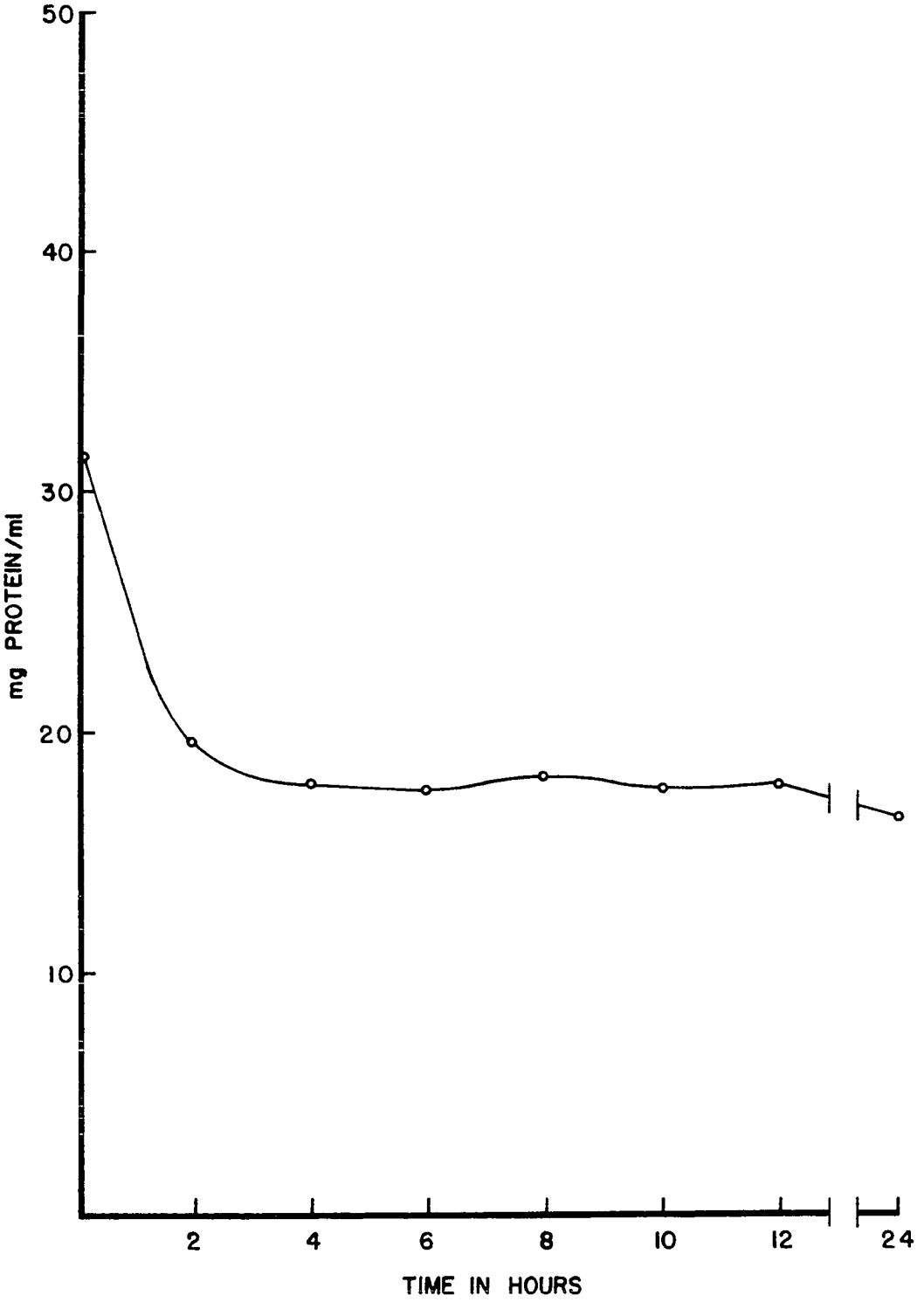

50
40
30
20
10
mg PROTEIN/ml
2
4
6
8
10
12
24
TIME IN HOURS

Figure 11. Amino nitrogen present in brain heart infusion as a function of time after inoculation.

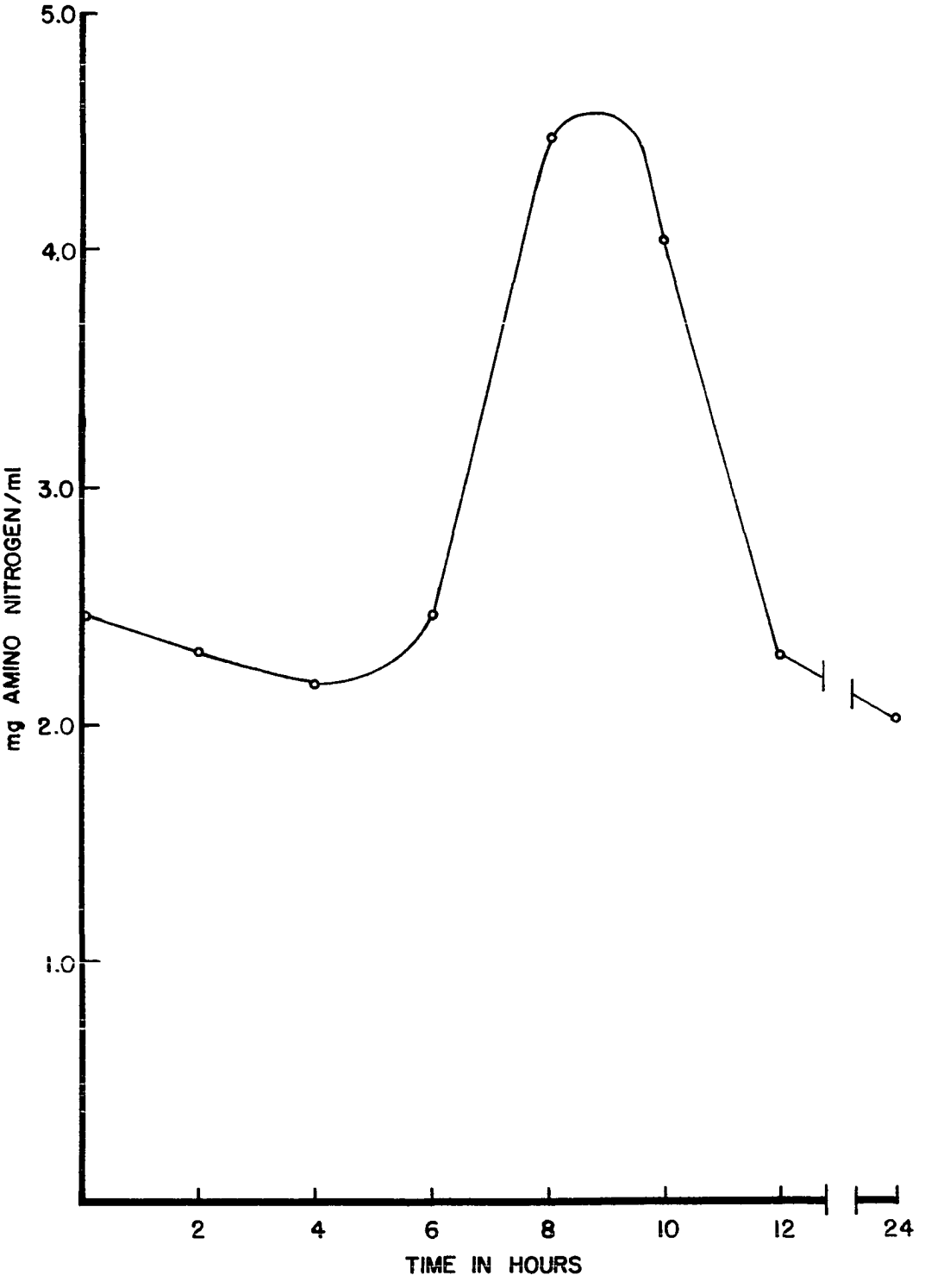

5.0
4.0
3.0
2.0
1.0
mg AMINO NITROGEN/ml
2
4
6
8
10
12
24
TIME IN HOURS

TABLE II

Free amino acids in pure and spent brain heart infusion broth at various times after inoculation.

		Relative amounts of each							
	Amino Acid	Pure	2	4	6	8	10	12	24 hr
1.	Aspartic	+++	++	++	++	+++	++	++	++
2.	Asparagine	++	+	+	+	+	+	+	-
3.	Alanine	+++	++	++	++	+++	++	+	+
4.	Diamino butyric?	-	-	-	-	*	+	-	+
5.	Glycine	+	+	+	+	+	+	*	-
6.	Glutamic	++++	++	++	++	+++	++	++	++
7.	Isoleucine	+	+	+	+	++	+	+	+
8.	Leucine	+	-	-	-	-	-	-	-
9.	Lysine	++	++	++	++	+++	++	+	++
10.	Phenylalanine	+++	+	+	+	++	+	+	+
11.	Serine	++	-	-	-	-	-	-	-
12.	Tryptophane	-	-	-	-	*	+	+	-
13.	Tyrosine	+++	++	++	++	++	++	+	+
14.	Threonine	+	-	-	-	-	-	-	-
15.	Valine	++	+	+	+	+	+	+	+
16.	Unknown	+	*	-	-	*	+	+	*

as diamino butyric acid appeared in the medium for the first time. From 10 hr to 24 hr relative amounts of many amino acids decreased.

These findings partially explain the results observed in Figure 11. Since no corresponding drop is detectable in the amount of protein, it is assumed that these amino acids do not appear as a result of breakdown of protein in the medium. The only other explanation is that they are released from the cells free amino acid pool or a result of cellular protein breakdown.

S. aureus is known to concentrate free amino acids (Gale, 1953; and Hancock, 1958). Using ^{14}C-labeled amino acids Gale demonstrated that washed cell suspensions were able to maintain a 300-fold concentration gradient of glutamic acid and other amino acids. A release of amino acids from the free amino acid pool would account for the observed increase of amino nitrogen at 8 hr (Figure 11). The reason why amino acids would be released and then taken back up again is obscure.

Amino acids which decreased in amount up to 6 hr were not necessarily those which appeared again at 8 hr. Leucine, serine, and threonine disappeared completely after 2 hr, and could not be detected in spent medium after this time. The relative amount of glycine remained constant through 10 hr, but glycine could not be detected in 24 hr spent medium.

Total organic matter is shown in Figure 12. There was a decrease in organic matter between 0 hr and 2 hr. At this same time glucose, and material measured as glycerol also declined (Figure 5 and Figure 7). From 2 hr to 6 hr there was an increase in organic material. This was followed by a decrease from the 6th hr to the 10th hr at which time an increase again occurred.

It has been reported that 10.3 mg of yeast extract or 5.8 mg of casein reduce one meq of dichromate (Johnson, 1949). From this determination it took 7.9 mg of brain heart infusion to reduce one meq of dichromate. Brain heart infusion is richer in organic matter than yeast extract, but not as rich as casein.

A correlation of Figure 12 and Figure 2 shows that as organic matter decreased resistance increased, and as organic matter increased resistance decreased.

Figure 13 and 14 show relative changes in pH and oxidation-reduction potentials during the growth of this organism. The pH continually decreased during the first 10 hr of growth reaching a pH of 6.2 at that time. No changes were observed from 10 hr to 12 hr. From 12 hr to 24 hr the pH increased reaching a maximum of 6.5.

The growth medium became continually reduced (drop in oxidation-reduction potential) during the first 12 hr of growth. The greatest reduction occured during the first 4 hr of growth. It was at this time that the largest production

Figure 12. Total organic matter in brain heart infusion broth as a function of time after inoculation.

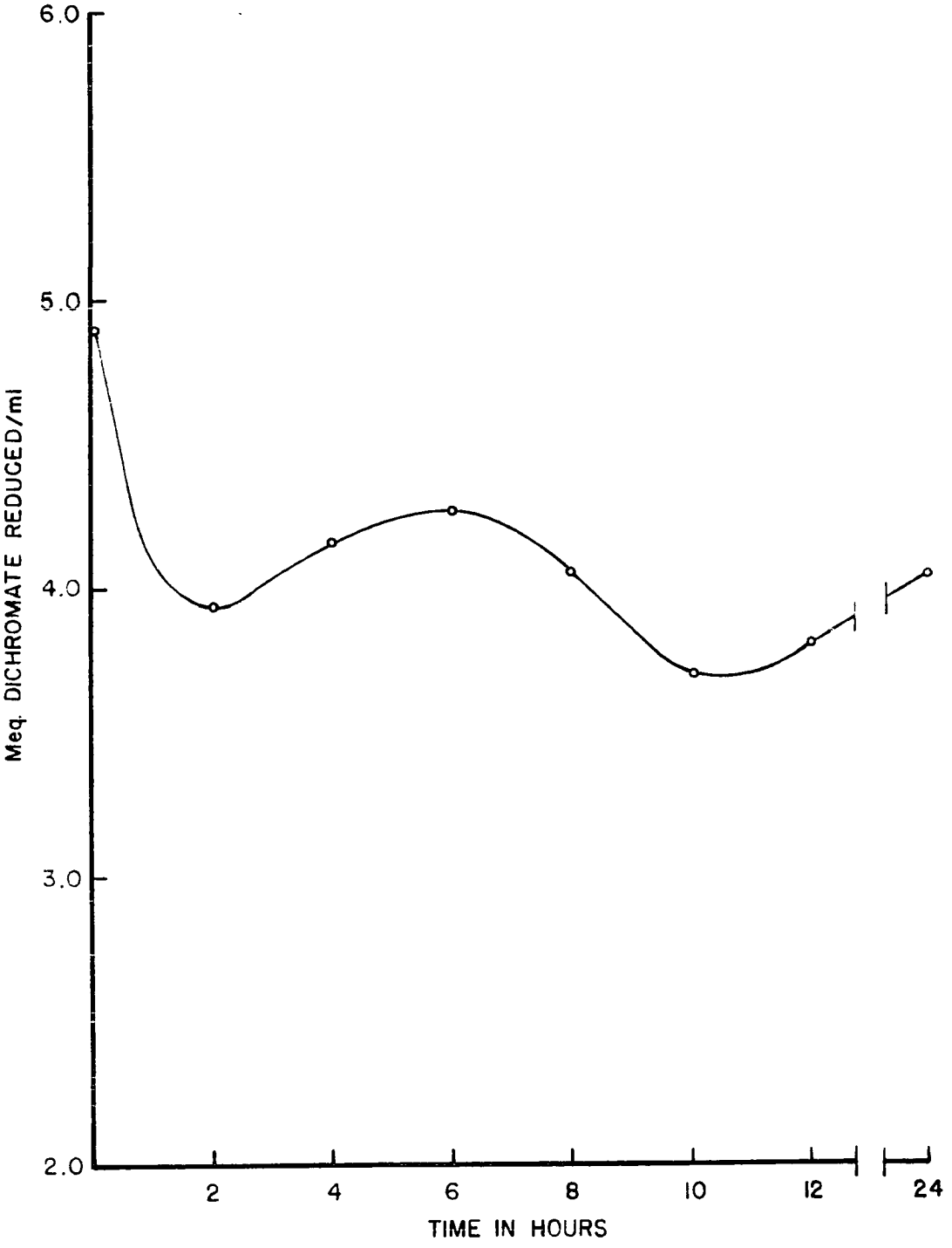

6.0
5.0
4.0
3.0
2.0
Meq. DICHROMATE REDUCED/ml
2
4
6
8
10
12
24
TIME IN HOURS

Figure 13. Changes in pH during the growth of Staphylococcus aureus in brain heart infusion broth as a function of time after inoculation.

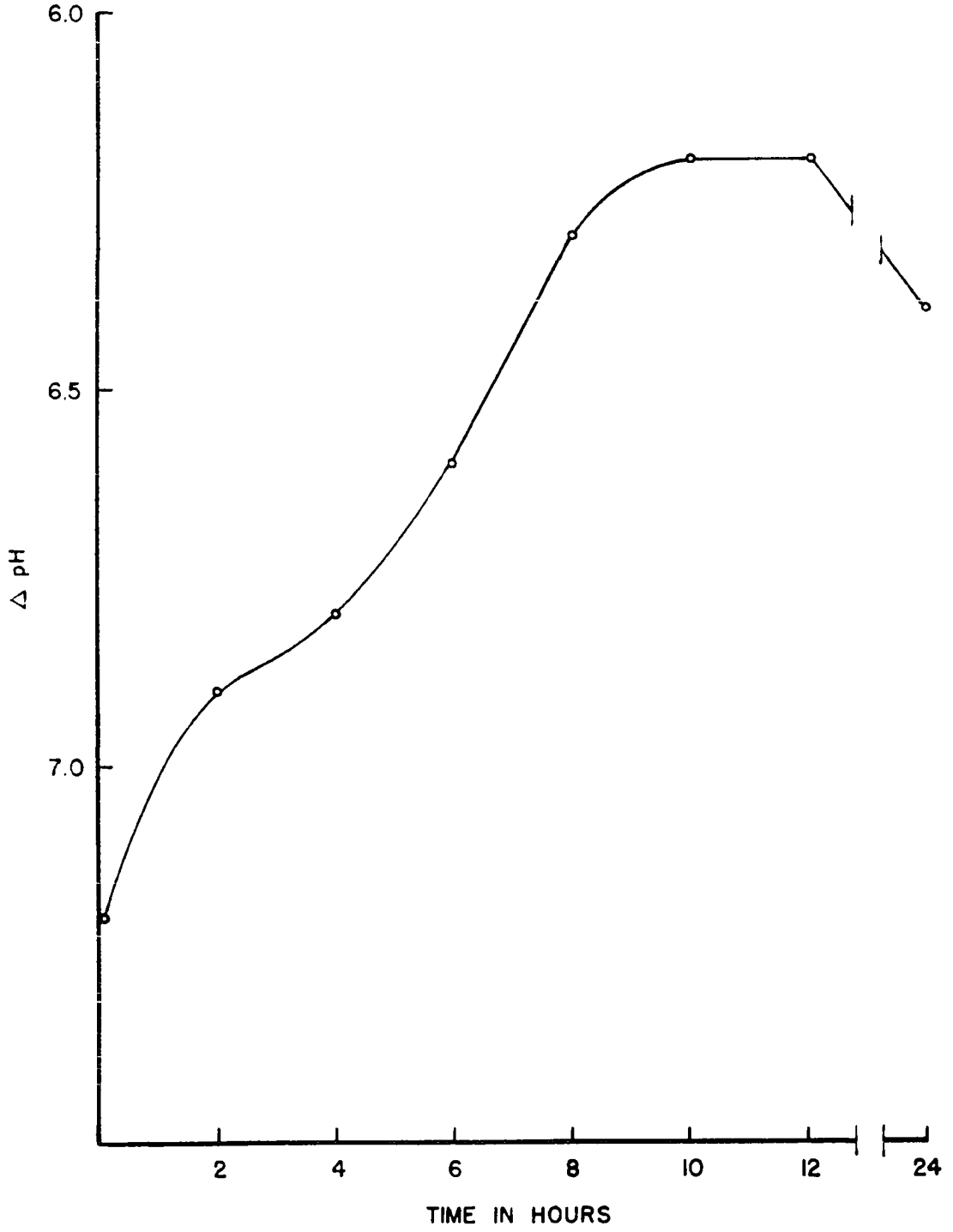
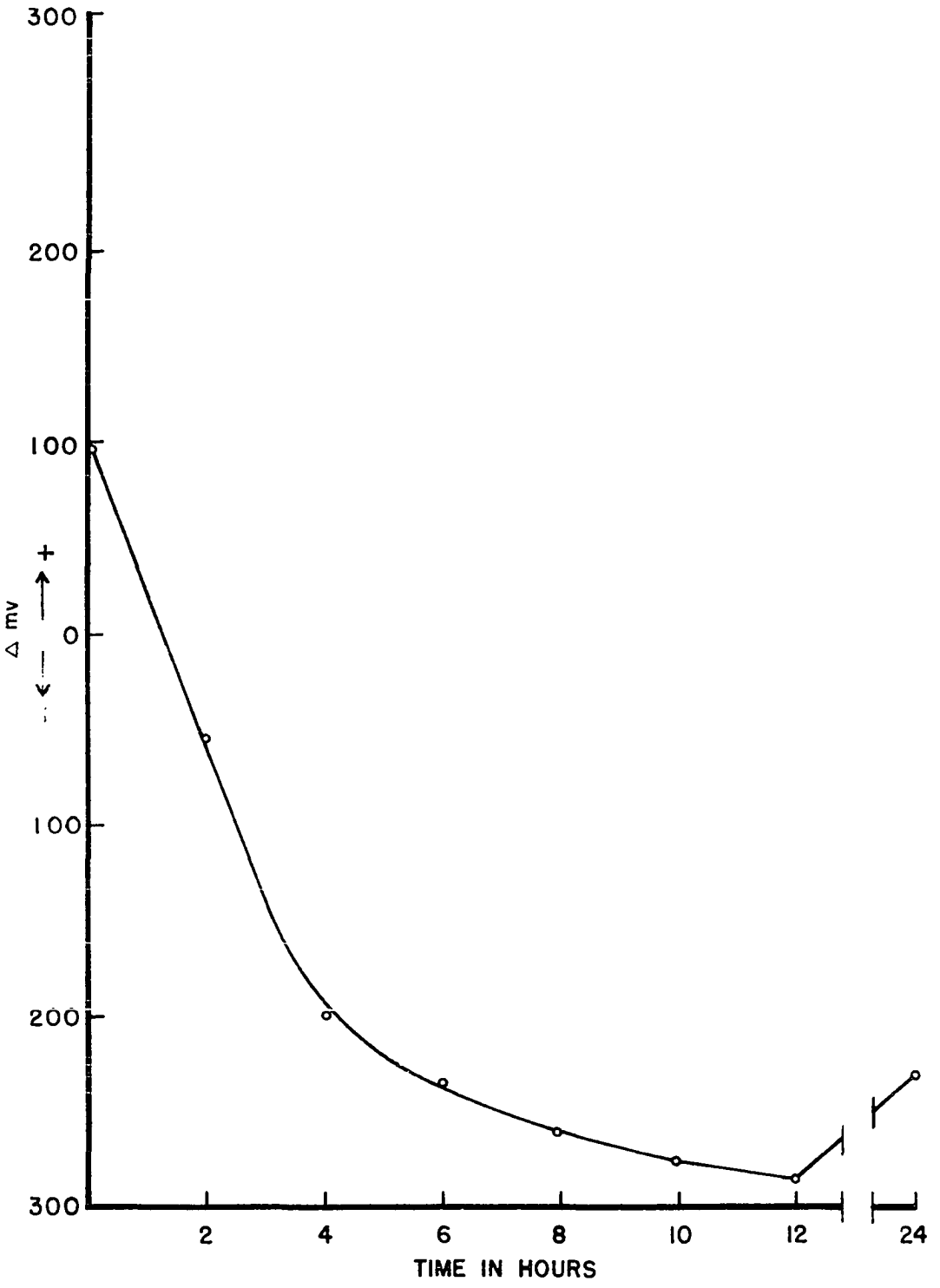

6.0
6.5
7.0
Δ pH
2
4
6
8
10
12
24
TIME IN HOURS

Figure 14. Relative changes in the oxidation-reduction potential during the growth of Staphylococcus aureus as a function of time after inoculation.


300
200
100
0
100
200
300
Δ mv
+
2
4
6
8
10
12
24
TIME IN HOURS

of acetic and lactic acid per unit time took place. It is, also, at this time that the largest QO_2 readings were observed. From 12 hr to 24 hr a slight oxidation (increase in oxidation-reduction potential) was observed.

The preceding data indicated that the environment was constantly changing throughout the growth cycle. In the first part of this work it was shown that by altering the environmental conditions the over all resistance to x-ray was affected. To determine further if changes in the environmental conditions are a reflection of changes in metabolism, and if there exists any correlation between changes found, and changes in radioresistance, metabolic studies were conducted on cells harvested at various times during the growth cycle.

Figures 15, 16, 17, 18, and 19 show changes in the QO_2 of various age whole cells. Intermediates of the tricarboxylic acid cycle, pentose cycle, glucose, pyruvate, lactate, and acetate were used as substrates. Endogenous respiration was substracted. The over all oxidative metabolism increased during the first 2 hr of growth reaching a maximum 2 hr after inoculation. This time period corresponds to the lag phase of growth (Figure 2). A maximum QO_2 rate at this time would be expected, since a great deal of synthetic activity occurs during the lag phase of growth.

As cells entered the log phase of growth the ability to stimulate oxygen uptake with a wide variety of substrates

Figure 15. QO_2 changes as a function of cell age after inoculation into brain heart infusion broth. Cells used for inoculation were 24 hr old, and are represented at 0 time.

(1) Glucose
(2) Lactate

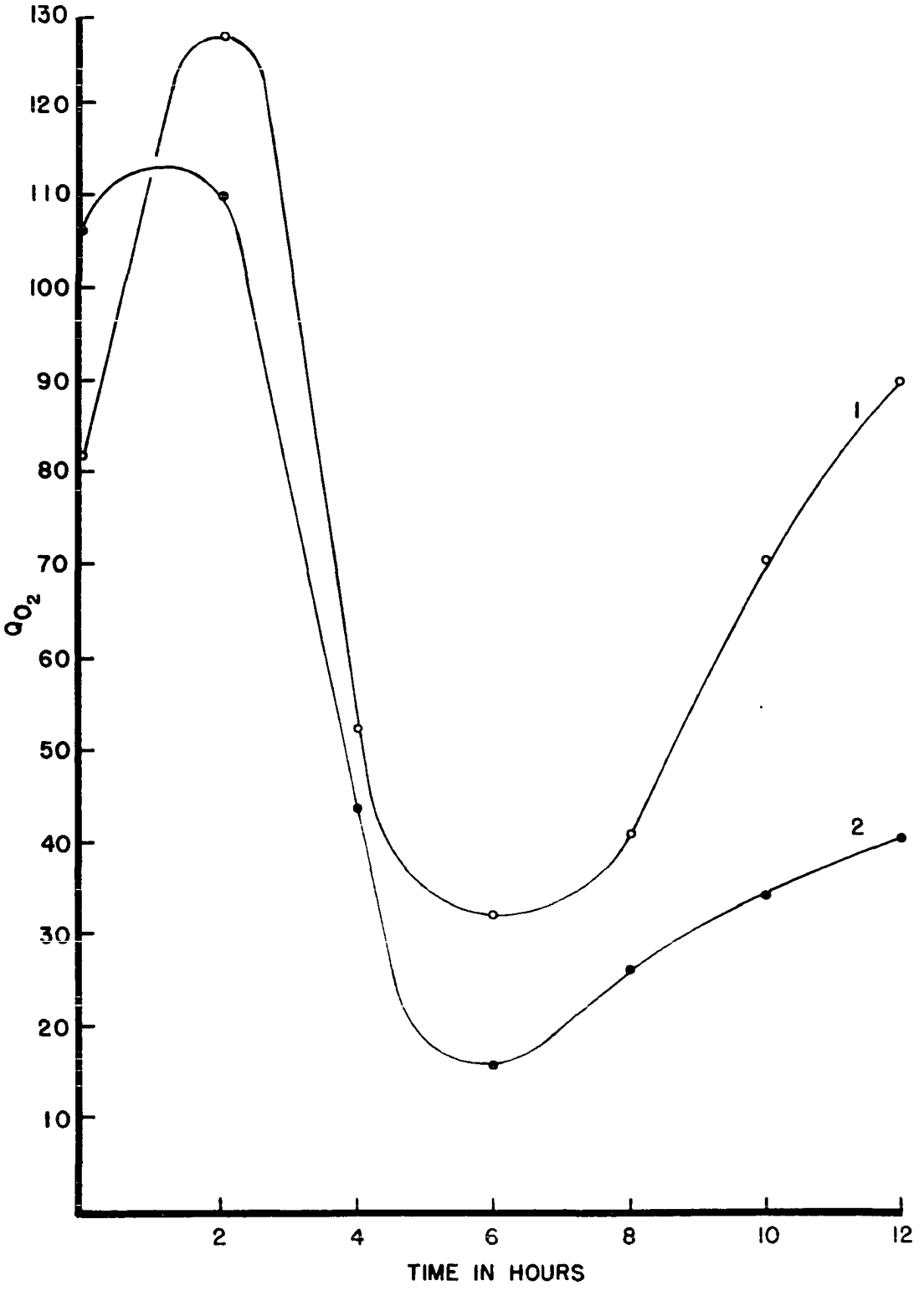

130
120
110
100
90
80
70
60
50
40
30
20
10
Q_{O_2}
1
2
2
4
6
8
10
12
TIME IN HOURS

Figure 16. QO_2 changes as a function of cell age after inoculation into brain heart infusion broth. Cells used for inoculation were 24 hr old, and are represented at 0 time.

(1) Pyruvate
(2) Acetate

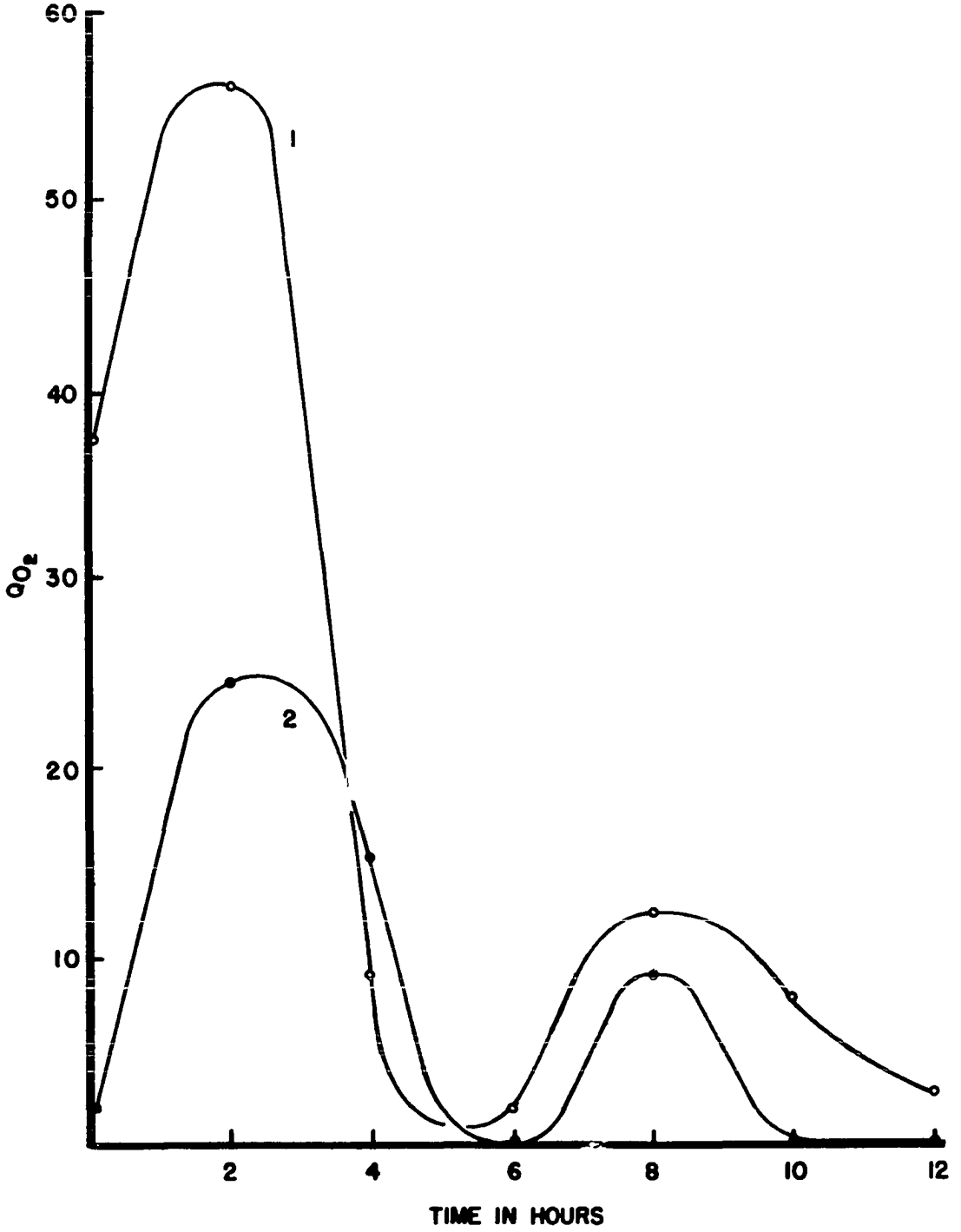

60
50
40
30
20
10
QO_2
1
2
2
4
6
8
10
12
TIME IN HOURS

Figure 17. QO_2 changes as a function of cell age after inoculation into brain heart infusion broth. Cells used for inoculation were 24 hr old, and are represented at 0 time.

(1) Succinate
(2) Citrate

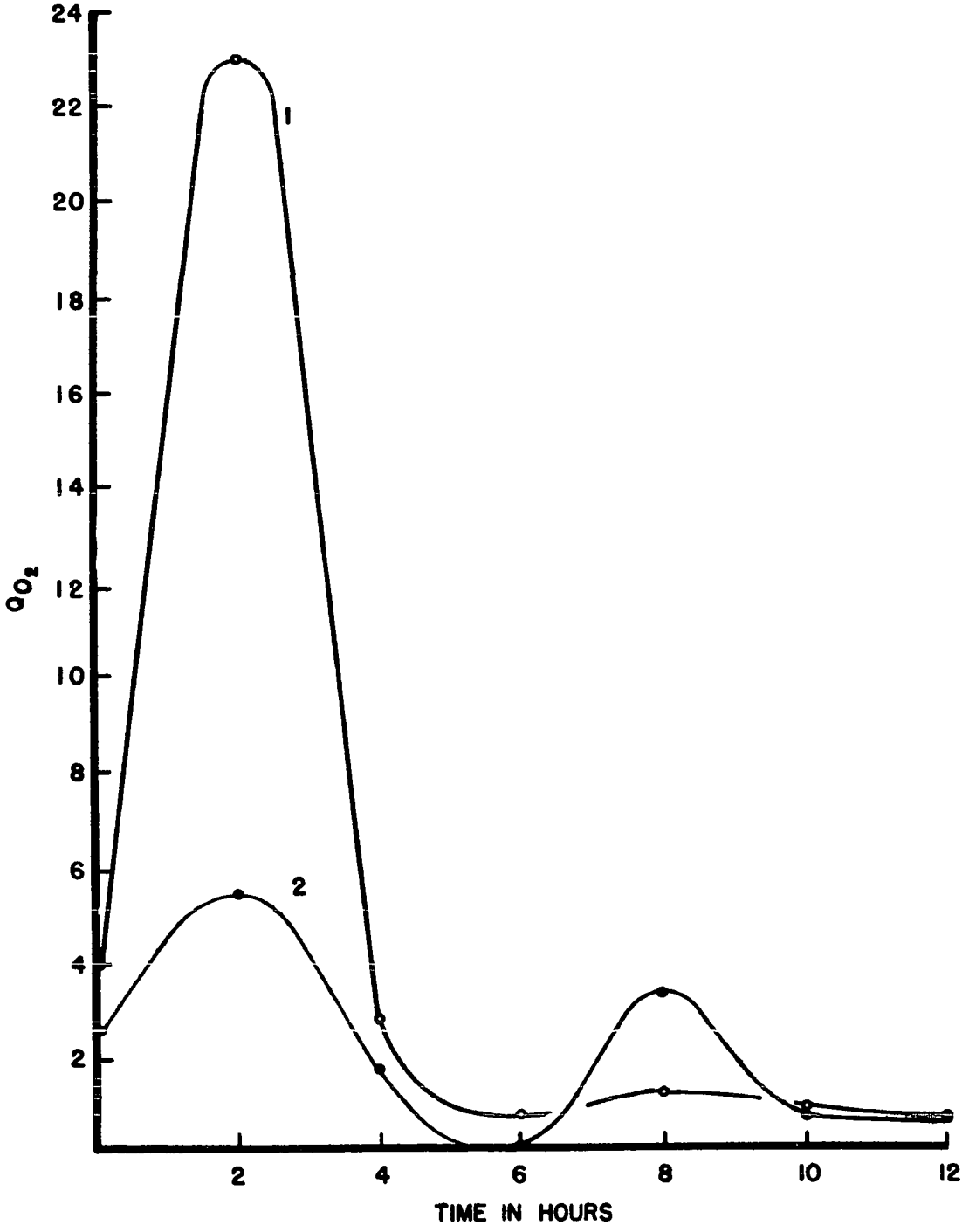

24
22
20
18
16
14
12
10
8
6
4
2
Q_{O_2}
1
2
2
4
6
8
10
12
TIME IN HOURS

Figure 18. QO_2 changes as a function of cell age after inoculation into brain heart infusion broth. Cells used for inoculation were 24 hr old, and are represented at 0 time.

(1) alpha-ketoglutarate
(2) Malate

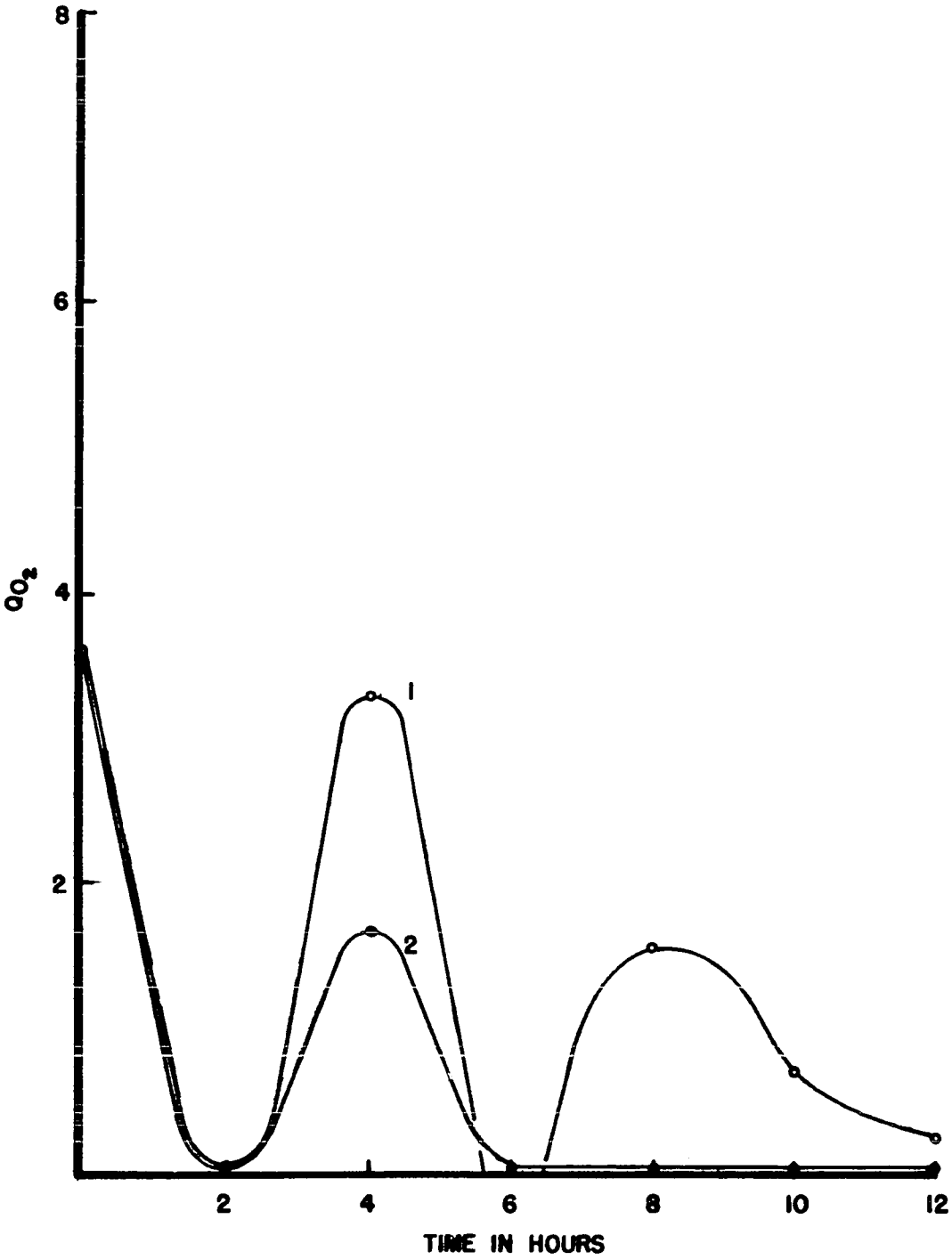
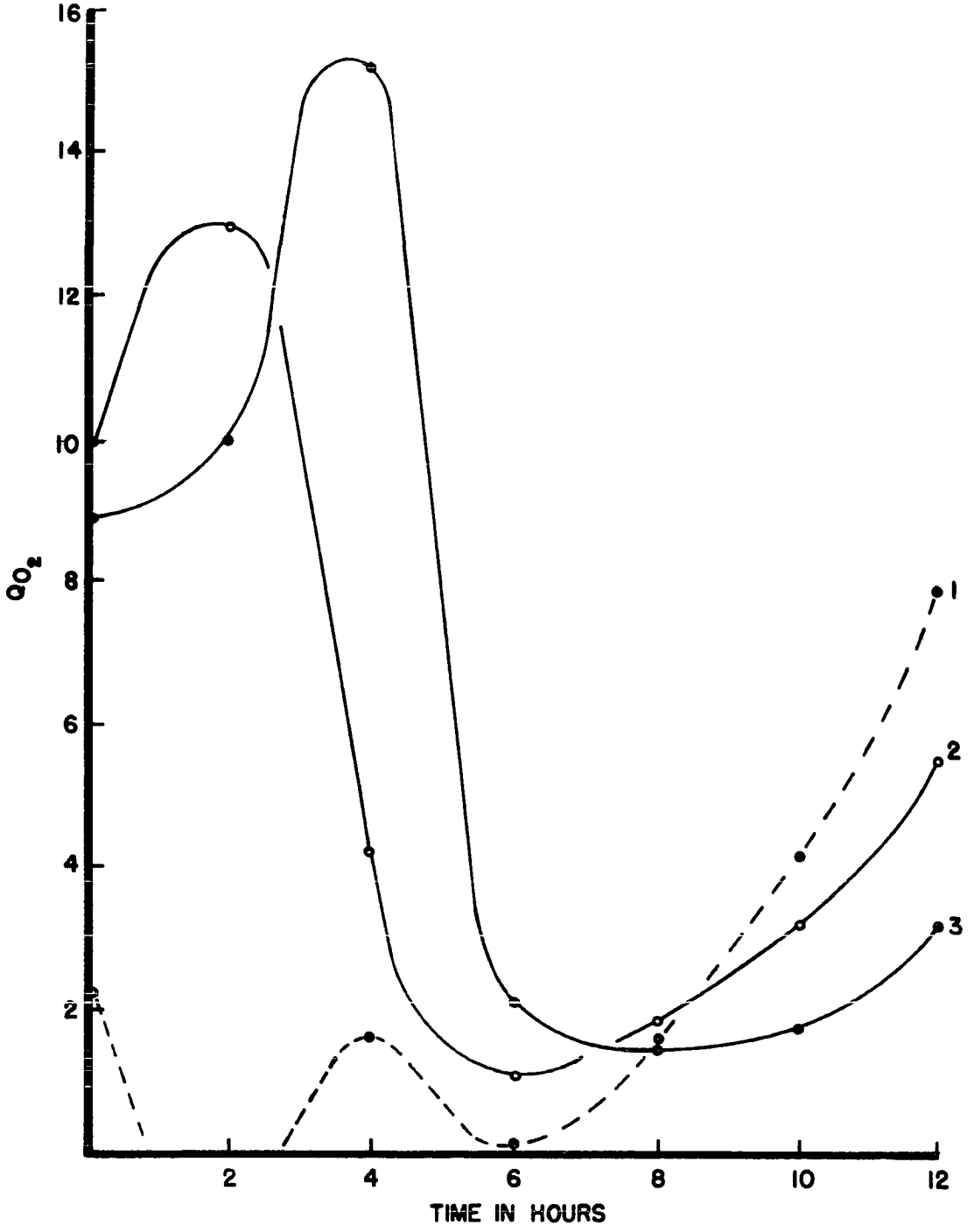

8
6
4
2
QO_2
1
2
2
4
6
8
10
12
TIME IN HOURS

Figure 19. QO_2 changes as a function of cell age after inoculation into brain heart infusion broth. Cells used for inoculation were 24 hr old, and are represented at 0 time.

(1) Ribose
(2) Gluconic acid delta lactone
(3) Gluconic acid


16
14
12
10
8
6
4
2
Q_{O_2}
1
2
3
2
4
6
8
10
12
TIME IN HOURS

decreased. This pattern continued until the late log phase of growth. As the culture entered into the stationary growth phase, between 6 hr and 8 hr, the oxidative metabolism again increased reaching a maximum at the time the stationary phase is reached. Except for oxidation of glucose, lactate, and pentose intermediates, the oxidative metabolism decreased as the stationary phase developed further.

The changes in respiration associated with the growth cycle are most likely a reflection of control mechanisms. Control mechanisms, operating at either the permeability or enzyme level or both, would account for the results observed. Both types of control have been demonstrated (Quastel, 1960; Magasanik, 1961; and Atkinson, 1965).

Similar variations in respiration, as a function of physiological age, have been demonstrated using muscle homogenates. The respiration was shown to greatly change depending upon the metabolic state of the muscle tissue (Krebs, 1959). Feedback control mechanisms were implicated as regulating respiration. As respiration increases, this creates conditions unfavorable for further progress and thereby causes the rate to slow down. This slowing down creates more favorable conditions and thus respiration increases.

The direct correlation between disappearance of glucose and glycerol from the medium (Figures 5 and 7), and changes in QO_2 values (Figures 15, 16, 17, 18, and 19) lends

support to the idea that control mechanisms are involved in the regulation of respiration. When glucose and glycerol utilization is rapid, QO_2 values are high. Likewise, when utilization of glucose and glycerol levels off, QO_2 values drop accordingly.

Table 3 shows the results obtained when ATP concentration in various age cells grown aerobically in brain heart infusion broth was determined. It is noted that although there is only a slight increase in the optical density during the first 2 hr of growth (lag phase), that ATP concentration essentially doubled. The ATP concentration in 5 hr old cells (middle log) was lower than in either 0 hr (24 hr old cells) or 2 hr old cells. At 8 hr (early stationary) the ATP concentration was again double that observed for 5 hr old cells, and at 10 hr the concentration decreased. Similar results have been reported for lag phase cells of Escherichia coli B/r (Billen et al., 1953). These workers found that during the lag phase of growth, there was a 6 fold increase in ATP concentration.

When increased QO_2 values were recorded, increased ATP concentrations were found. Likewise, it was at these times that utilization of glucose and glycerol and resistance to x-ray were the greatest.

In analyzing these results it can be seen that the oxidative metabolism of cells in various phases of growth is not constant. No direct correlation can be seen between

TABLE III

Changes in the relative amounts of ATP/mg dry wt during the growth cycle of Staphylococcus aureus grown aerobically in brain heart infusion broth.

Cell age	Relative amount	Optical density
*0 hr	7.52	0.17
2 hr	13.20	0.175
5 hr	5.28	0.45
8 hr	12.28	0.82
10 hr	6.24	0.85

*24 hr old cells used as inoculum

accumulation or disappearance of any metabolic product which corresponds with resistance or sensitivity of cells to x-radiation. This would be expected, since metabolic products produced under different environmental conditions would vary, and in turn would cause the place of occurrence of resistant and sensitive populations during the growth cycle to vary. This has been shown not to be the case (Figures 2, 3, and 4). The rate of disappearance of glucose and glycerol does correlate with increased or decreased resistance, but glucose is not present in nutrient broth. The one process which does correlate very well with increased or decreased resistance is rate of metabolism. Everytime resistance increased, the rate of metabolism increased (0 hr to 2 hr and 6 hr to 8 hr). Likewise, as the rate of metabolism decreased, resistance decreased.

Oxidative metabolism via cytochromes, with oxygen being the final hydrogen acceptor, could not account for increased resistance, since under complete anaerobic conditions no oxygen is present, and a cytochrome system similar to that in aerobic systems is not thought to exist (Newton and Kamen, 1961). Some result of increased oxidative metabolism or anaerobic fermentation would therefore be suspected. One effect which is very likely is increased electron transport which would result in an increased production and accumulation of adenosine triphosphate (ATP) similar to that shown in Table 3. This is more meaningful, since a

partial check of carbon dioxide production in late lag, middle log, and early stationary phase cells grown anaerobically showed essentially the same increases and decreases. Glucose was used as a substrate in these determinations.

It has been reported that growth conditions after x-radiation greatly alter the survival of an organism (Stapleton, Sbarra, and Hollaender, 1955). These workers observed using E. coli B/r that when growth was on a complex medium before x-irradiation, and plated on minimal medium after x-irradiation, that the organism showed more inactivation than if plated on a complex medium after irradiation. They suggested as a possible explanation that cells grown on complex media lack certain enzymes needed for growth on minimal, and that radiation inhibited the ability to form these enzymes.

Table 4 shows the results from similar experiments using S. aureus. It can be seen that increased or decreased resistance can be demonstrated by altering the environmental conditions in different aerobic systems. These variations are not due to use of cells of different physiological age, since all experiments were conducted using cells harvested at 60% to 70% maximum growth. This corresponds to the late log phase of growth. It should be pointed out that maximum cell numbers varied with the medium used, but by making appropriate dilutions approximately the same number of cells were irradiated in each case.

TABLE IV

Effect of pre- and postirradiation medium on the survival of Staphylococcus aureus after 4500 r of x-radiation.

	Preirradiation medium	*Postirradiation medium	Percent Survivors
1.	nutrient broth	nutrient agar	32
2.	BHI broth	BHI agar	48
3.	synthetic broth	synthetic agar	34
4.	BHI broth	nutrient agar	20
5.	BHI broth	synthetic agar	10
6.	synthetic broth	BHI agar	38

BHI = brain heart infusion
*Medium used for growth of controls and x-rayed cells.

Under conditions no. 1 and no. 2 (Table 4), survival was essentially the same as that shown in Figures 2 and 3. Cells grown in a synthetic broth and plated on synthetic medium plus agar, showed a survival similar to that observed under conditions no. 1 (Table 4). When the growth and plating medium differed, survival was greatly affected (no. 4 and no. 5, Table 4). Under conditions no. 6 (Table 4), this effect was not observed. The results obtained in this series are similar to those reported by Stapleton, Sbarra, and Hollaender (1955).

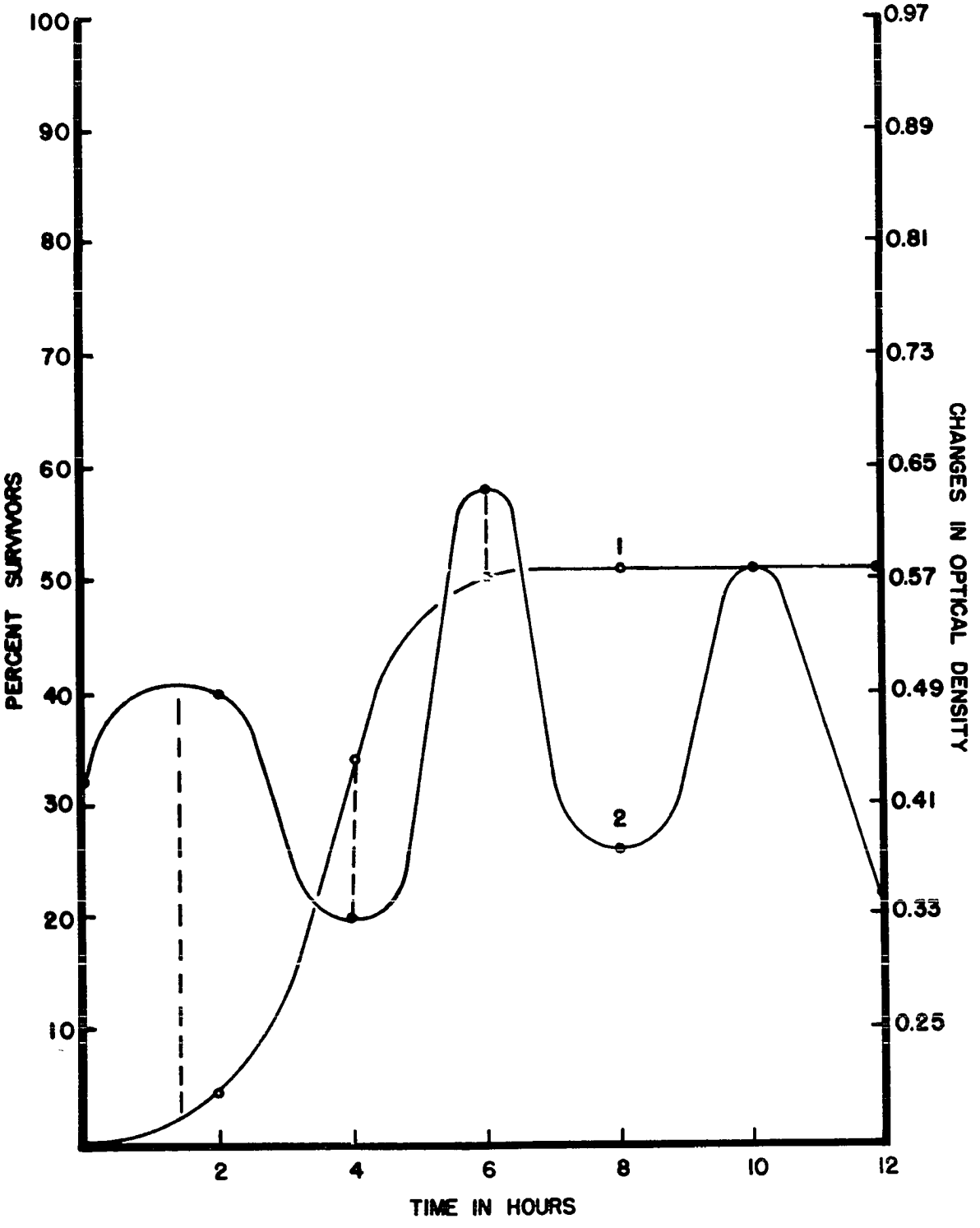
To determine to what extent similar conditions affected sensitivity during the life cycle, alterations of survival conditions were conducted. Figure 20 shows the results obtained when cells were grown anaerobically, irradiated anaerobically, and allowed to recover aerobically. Aerobic recovery of anaerobically grown cells increased the total sensitivity, but not the shape of the survival curve as a function of cell age. Relative changes in the various maxima were affected as was the case observed in Figures 2, 3, and 4.

When growth and recovery were anaerobic (Figure 4) the survival of late lag phase cells was 95%, and early stationary phase cells 70%. Where aerobic recovery was allowed these values were 40% and 60% respectively. This indicated that any alteration in recovery procedure has its greatest affect on cells in the lag phase of growth.

To learn more about this combined lethal effect of

Figure 20. Effect of aerobic recovery on the sensitivity of previously anaerobically grown cells as a function of cell age. Irradiation was conducted under anaerobic conditions.

Growth medium was brain heart infusion. Controls and irradiated samples were plated on brain heart infusion agar. (1) Changes in optical density corresponding to growth. (2) Sensitivity changes as a function of cell age following a dose of 4500 r.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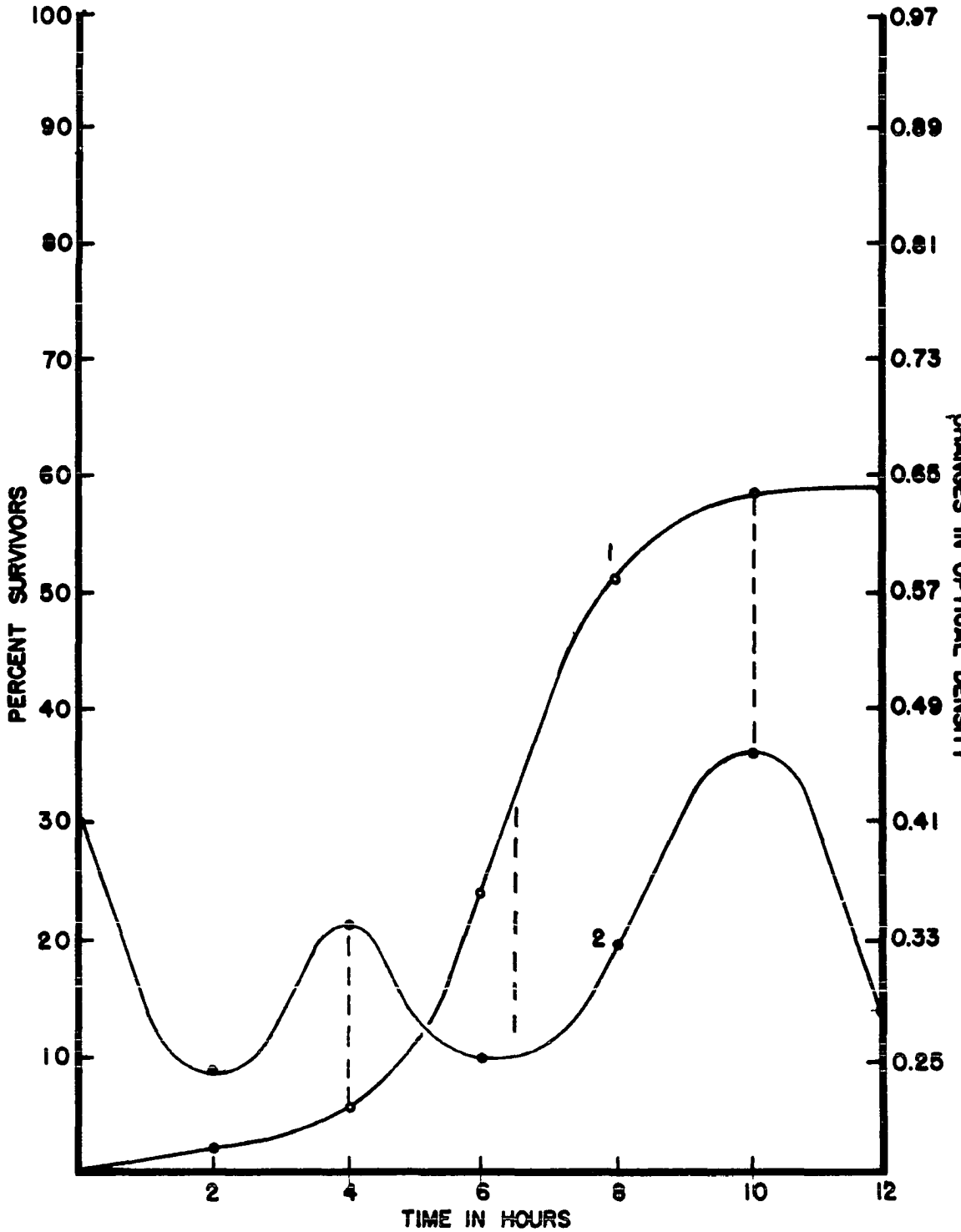
adaptation and radiation, studies on the effects of preadaptation were conducted. The results of the first of this series is shown in Figure 21.

When a previous aerobically grown culture was used as inoculum in an experiment as described above, sensitivity during the early part of the lag phase decreased. It is noted that the lag phase under these conditions was longer than that observed when only one adaptation takes place (Figure 20). It is also seen that the resistance was high in the late lag and early stationary phase cells. It would appear that the adaptation itself increased the sensitivity to radiation. Only when the adaptation was fully expressed (at late lag phase) did the cells start to become more resistant. If radiation inhibited the adaptive process this would not be expected to be the case. Previous aerobically grown cells incubated 2 hr under anaerobic conditions would be expected to have less difficulty in readapting to aerobic survival conditions than would cells grown 8 hr under anaerobic conditions if the adaptive processes were inhibited, and this was not what was observed.

One can not rule out the possibility of preferential adaptation over repair. Under conditions where growth of an organism is blocked by the lack of some nutrient, which occurs when transferred from a complex medium to a synthetic medium, synthesis of new enzymes must take place. These are sometimes preferentially synthesized (Pardee, 1955; Rickenberg and

Figure 21. Effect of preadaptation on radiosensitivity as a function of cell age. An aerobically grown culture was used as inoculum. Preirradiation was anaerobic; irradiation was anaerobic; and recovery was aerobic.

Growth medium was brain heart infusion. Controls and irradiated samples were plated on brain heart infusion agar. (1) Changes in optical density corresponding to growth. (2) Sensitivity changes as a function of cell age following a dose of 4500 r.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and Lester, 1955). Also, it is possible that the process of adaptation exposes more damageable sites. Either of these could account for the results observed.

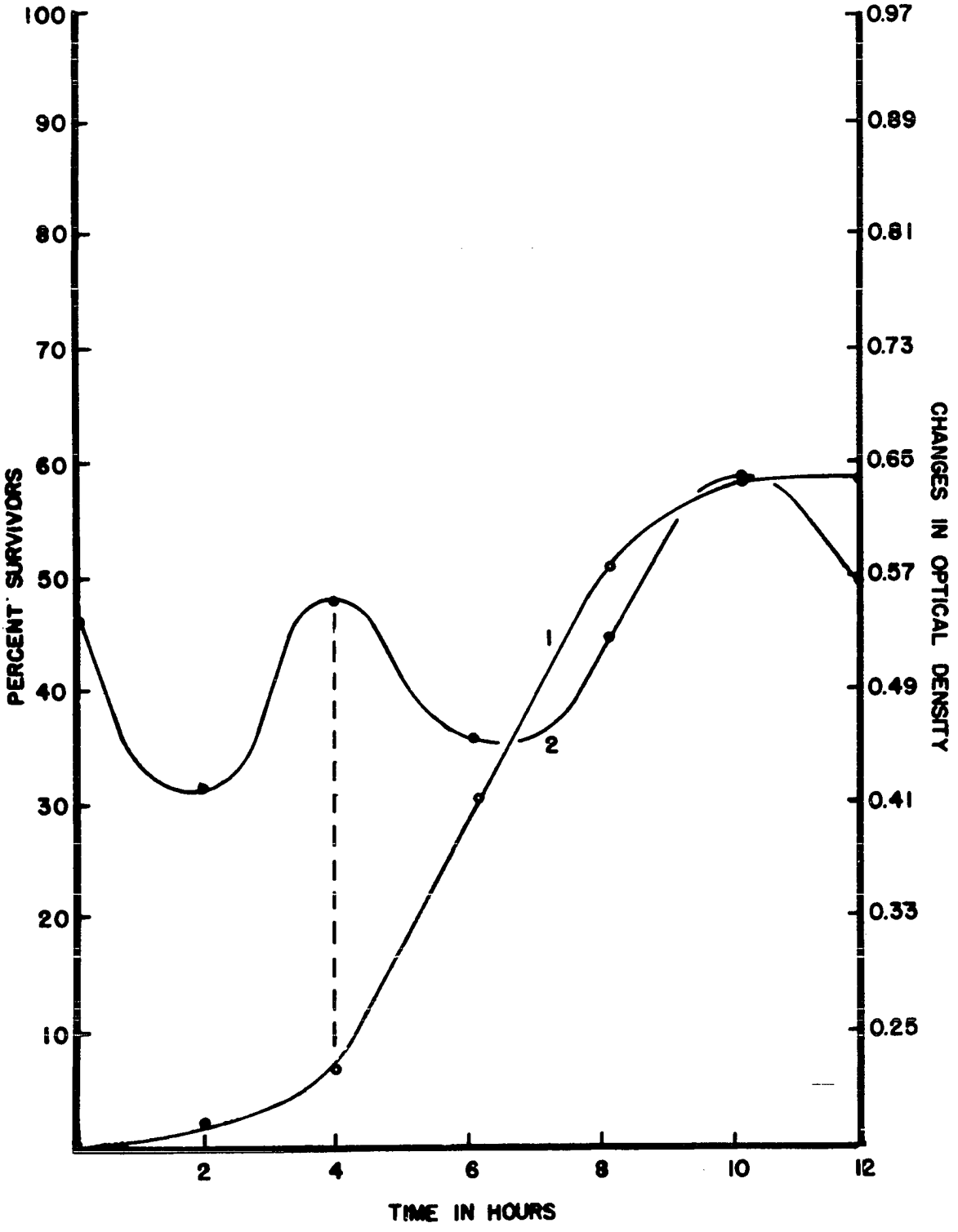
Figure 22 shows similar results when cells were previously grown on brain heart infusion; transferred to synthetic medium; and plated on brain heart infusion. The same drop in resistance during the early lag phase was observed here that was observed under conditions in Figure 21.

To learn more about the effect conditions during irradiation played in altering the sensitivity of cells to x-radiation, samples were irradiated at different temperatures under aerobic conditions. Only a slight increase in sensitivity was noted when cell suspensions were irradiated at a temperature of 30 C as compared to 5 C.

All these data strongly indicate that survival is directly dependent on growth conditions before x-ray, and that the more homogeneous the growth conditions, the greater the survival.

Figure 22. Effect of preadaptation on radiosensitivity as a function of cell age. A culture previously grown on brain heart infusion was used as inoculum. Preirradiation growth medium was synthetic, and postirradiation medium was brain heart infusion agar. Growth, irradiation, and recovery were aerobic.

(1) Changes in optical density corresponding to growth. (2) Sensitivity changes as a function of cell age following a dose of 4500 r.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CHAPTER IV

DISCUSSION

From these data it is obvious that environmental conditions are very important determinants of x-ray sensitivity. Such conditions have also been shown to be important in determining sensitivity to ultraviolet light and heat (Stapleton and Engel, 1960).

Alterations in metabolism occur when the environment is changed (Herbert, 1961; Pardee, 1961; and Kornberg, 1965). These data show that great variations in metabolism take place during the growth cycle. This would be expected, since the environment to which cells are exposed while growing in a culture flask of any medium is constantly changing.

It has been stated by Herbert (1961) that "there are few characteristics of micro-organisms which are so directly and so markedly affected by the environment as their chemical composition." This is so much the case that it becomes virtually meaningless to speak of the chemical composition or metabolism of an organism without at the same time specifying the environmental conditions that produced it. Likewise, the same can be said for studies on the effects of radiation on living systems.

Many workers who study the effects of radiation make little effort to describe the environmental conditions under which their results were obtained. Many metabolic studies on resting cells are conducted each year, and statements made to the effect that an organism does, or does not, possess certain metabolic capabilities. Many studies of this type are unfortunately invalidated. From these data it is seen that oxidative metabolism, lactic acid production and many other processes can change in a period of 2 hr. Therefore, comparison of work where environmental growth conditions are not the same have little meaning.

The place of appearance of various resistance maxima and minima directly associated with the growth cycle were not affected by environmental conditions. In every case studied, there was always an increase in resistance in late lag and early stationary phase cells. This indicates that some event in common with all cell populations in these phases must occur.

The only metabolic function found in common with these phases was an increased rate of metabolism accompanied by an increased intracellular level of adenosine triphosphate (ATP). It would be expected that this feature would be common to both increased aerobic respiration and anaerobic fermentation. The increased ATP level directly correlates with increased resistance.

Adenosine triphosphate would be expected to assist

in repair of x-ray damage. Injection of ATP has recently been shown to protect against radiation induced chromosome loss in Drosophilia (Mittler and U, 1966). This protective effect of ATP could be demonstrated if injected immediately before or after administration of 2000 r of x-ray, and these workers concluded that the ATP was not acting as a radiation shield but was aiding in the repair process.

If radiation either temporarily or totally damaged the mechanism involving ATP production or if repair of any damage had to take place within a short period of time, then the amount of repair requiring energy would be directly dependent on ATP levels existing in the cell at the time of treatment.

The first evidence that ATP forming mechanisms were possible sites of radiation damage came in 1952. Reduced P/O ratios were found in spleen mitochondria from rats which had received 800 r 1 hr previously. Since that time numerous other workers have observed similar effects (Ord and Stocken, 1961). The drop in ATP levels observed was not due to release of ATPase since when the activity of this enzyme was inhibited with fluoride, reduced phosphorylation was still detected.

Van Bekkum (1956), using succinate as a substrate, found an increased dependence for exogenous cytochrome c after irradiation of rat spleen mitochondria. Cytochrome c increased the oxygen uptake of normal spleen mitochondria, but the effect was much greater with those from irradiated animals.

This increased oxygen uptake was detected 30 min and 1 hr postirradiation and was marked 2 hr after a dose of 700 r. This cytochrome c effect which he found very soon after exposure may reflect direct damage to the mitochondrion with consequent reduction in oxidative phosphorylation.

In earlier studies with Staphylococcus aureus, oxygen uptake was found to be reduced by irradiation, and at the same time there was an increased dependency on an external hydrogen acceptor. No difference in the activity of irradiated and non-irradiated enzymes could be found, and damage to the cytochrome system was proposed as the cause of these observed results (Howell, 1964).

It appears that some radiation lesions must be repaired before cell division takes place. Stapleton et al., (1953) showed that survival of Escherichia coli B/r following x-ray treatment was increased by lowering the postirradiation incubation temperature to about 18 C. Jagger (1964) proposed that the mechanism of photoprotection against ultraviolet damage involves delay of cell division after irradiation. Findings by Savage (1966) indicate that this may also be the mechanism involved in photoprotection against x-ray damage.

Since a great deal of information exists indicating that processes involving coupled phosphorylation are radiosensitive, and that certain radiation lesions require rapid repair, the survival of the organism would be directly dependent upon the energy available at the time of treatment

to make such repairs.

From these data it would appear that the mechanisms involved in coupled phosphorylation under anaerobic conditions would have to be either more resistant or fewer types of damage would have to occur so that less energy would be needed for repair.

As stated in the results, the process of adaptation itself made the cells more sensitive to x-rays. A possible explanation was preferential adaptation over x-ray damage repair. A more likely explanation is that the combined effect is more lethal.

The process of adaptation and x-ray damage repair would put an increased drain on cellular ATP levels, and if coupled phosphorylation was inhibited or partially impaired, repair of any x-ray damage would be delayed if cellular ATP levels were low.

Increased ATP concentrations would account for the findings of Stapleton and Engle (1960) that when cells were most resistant to x-irradiation, they were also more resistant to ultraviolet light and heat.

The more homogeneous the environmental conditions, the more resistant cell populations are to the detrimental effects of x-ray. The role which cell metabolism plays in this can not be overlooked. Since the overall sensitivity is directly dependent upon growth conditions prior to treatment, a study of differences in metabolism in different environments would seem very meaningful.

CHAPTER V

SUMMARY AND CONCLUSIONS

Definite specific changes in radiosensitivity were found to occur during the growth cycle of Staphylococcus aureus. These changes could be directly associated with the various phases of growth, and the phase of growth where these changes occurred was not affected by altering the environmental conditions. With cultures grown on various media, and under aerobic or anaerobic conditions, increased resistance was always associated with the late lag and early stationary phases of growth.

Large variations in metabolism of this organism were demonstrated throughout the growth cycle. Oxidative metabolism was highest during the lag phase of growth; dropped off during the log phase; increased again during the late log phase; and decreased during the early stationary phases.

Lactic and acetic acids were produced during the first 6 hr of growth with maximum production between 2 hr and 4 hr after inoculation (late lag and early log). After this time production decreased, and from 6 hr to 10 hr these acids were utilized. Maximum utilization occured at 10 hr after which time production again occured.

Glucose and glycerol disappearance correlated with increased oxidative metabolism. These compounds disappeared at a rapid rate from the early to the late lag phase of growth. During the first part of the log phase of growth, utilization levels off. As the cells entered into late log phase, a rapid increase in rate of disappearance again occured. This increased rate lasted until cell populations reached early stationary phase at which time a decrease was observed.

Increased oxidative metabolism and increased utilization of glucose and glycerol were always accompanied by increased intracellular levels of adenosine triphosphate (ATP).

Of all metabolic functions studied, only increased rate of metabolism directly correlated with increased resistance during the late lag and early stationary phases of growth. In the aerobic and anaerobic systems studied, the rate of oxygen uptake and carbon dioxide production was always higher in resistant cell populations than sensitive cell populations. Also, it was at this time that glucose and glycerol utilization, lactic and acetic acid production or utilization were highest, and increased levels of ATP were observed.

It was concluded that the increases in resistance directly associated with the various phases of the growth cycle are the result of increased metabolism accompanied by increased intracellular levels of ATP.

The direct correlation between ATP levels and radiation resistance during the growth cycle made it possible to

conclude that repair requiring energy is directly dependent upon the concentration of this compound at the time of damage. A partial block or inhibition of coupled phosphorylation could well account for this dependency. This may well be the case, since decreased oxygen uptake and increased dependency on an external hydrogen acceptor following irradiation has been demonstrated previously in this organism (Howell, 1964).

The relative amounts of deoxyribonucleic acid per cell in resistant and sensitive cells could not be shown to account for the results observed. Likewise, mutant formation can not account for these results, since the differences in percent between resistant and sensitive cells were higher than would be expected if this were the case.

The overall sensitivity of cells in any phase of growth was greatly modified by alterations in the environment. Maximum resistance was observed when the pre- and postirradiation environmental growth conditions were the same. Variations in heterogeneity and homogeneity of environment had its greatest effect on lag phase cell populations. Inhibition of the adaptation processes would be suspected, but it was found that the adaptive processes were not inhibited by low doses of x-radiation. This was determined by preadaptation studies.

It was shown that cells irradiated during the process of adaptation were more sensitive to radiation than those which were irradiated after adaptation. This was shown for

adaptations from aerobic to anaerobic, anaerobic to aerobic, and from growth on a complex medium to growth on a synthetic medium. It was concluded that the preadaptation lowered the ATP concentration in the cells thereby rendering them more sensitive to x-radiation.

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