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THE PHYSIOLOGICAL BASIS FOR THE INACTIVITY OF CITRATE OXIDASE IN *ESCHERICHIA COLI* AND *BRUCELLA ABORTUS*

by

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The apparent inability of intact cells of *Brucella abortus* and *Escherichia coli* to oxidize citrate, whereas cell extracts do oxidize it, has been noted frequently and is representative of a general phenomenon of apparent enzyme latency *in situ*. The present study attempts to explain this anomaly.

The oxidative response of *B. abortus* to citrate under varying conditions was investigated by means of conventional manometric techniques. It was found that neither varying pH and concentration of citrate, addition of various energy sources, growth conditions or age of cells, addition or removal of metallic ions, use of citrate analogs, freezing, drying, nor treatment of cells with various solvents had any effect in enabling the cells to measurably increase their rate of oxygen uptake in the presence of citrate. However, cell extracts did contain an active citric oxidase and large masses of cells gave a measurable increase in the rate of oxidation of citrate. Furthermore, isocitric acid lactone appeared to be oxidized readily. It was concluded that

citrate is degraded by intact B. abortus, although at a rate too low to be detected by ordinary methods in the presence of its normally high endogenous rate of respiration.

The oxidative response of E. coli to citrate was not altered by the addition of variously substituted esters of citric acid, nor by treatment of the cells with various solvents. However, cell rupture, drying, treatment with lysozyme, or heating for short periods at 100°C revealed an active citric oxidase in E. coli.

The penetration of citrate into B. abortus and E. coli was measured by a direct method which is based on the theory that large masses of cells will act as a diluent for any substrate which penetrates them and that the degree of penetration will be directly proportional to the dilution. Thus,

$$Scit = \frac{Vs}{Wc} \left(\frac{Co}{Cf} - 1\right) \times 100$$

where Scit = percentage of cell pack penetrated by citrate, Vs = volume of substrate, Wc = weight of packed cells, Co = initial concentration of substrate, and Cf = final concentration. The volume of the intercellular space was determined by a similar formulation using a non-penetrating substrate (inulin) and was substracted from Scit to give the corrected citrate space (R).

Citrate was analyzed by a pentabrom acetone method and inulin, gravimetrically.

Thus it was calculated that citrate entered 41% of a B. abortus cell (in 10 min at pH 6.4 and 0°C) and 23% of an E. coli cell under the same conditions. The space in E. coli penetrated by citrate was not changed by the addition of glucose or isocitrate nor did it decrease with increased citrate concentration. The space is subject to variables of temperature (0° = 23%; 60° = 40%), pH (pH 9.0 = 15%; pH 3.0 = 70%), and ion concentration. In the absence of ions, the space penetrated was 12%, but this increased with ion concentration up to a maximum (40%) in 0.100 M sodium or potassium ions. Calcium or magnesium had a maximal effective concentration of 0.050 M. If present at a concentration over 0.0125 M, ferric ions caused complete disappearance of the added citrate. A small amount of calcium appeared to be essential for maximum penetration of citrate. Dried cells had 50% of their volume available to citrate. Treatment of cells with butanol, chloroform, or toluene similarly caused a marked increase in citrate space (up to 75%).

It was concluded that the inability of intact *E. coli* to oxidize exogenous citrate occurs either because of impermeable membranes interior to the cell surface, or because of a labile inhibitor of the oxidase within the cell.

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INTRODUCTION

One of the most tenacious barriers to knowledge of cellular function is the phenomenon of permeability. The subject has been reviewed frequently (cf. Daveson,[21]) with reference to higher plants and animals, but not until recently has any concerted effort been made to unravel the mysteries of permeability in bacteria. Recent advances in the study of cellular structure and metabolism no longer make it possible to neglect investigation of the role of semi-permeable structures either at the surface or in the interior of these cells.

It has been the popular custom in metabolic investigations to study the cell first in the intact state and then to break it up and study the homogenate. In general the same enzymes were found by either technique, but occasionally the broken cell revealed enzymes unsuspected in the intact cell. Such observations were conveniently attributed to permeability, "and for the biochemist the invocation of this magic word puts an end to further thought about the matter"[86].

Consequently it is generally assumed that the intact surface membranes of the cell exclude the particular substrate in question. This is supported by the fact that endogenously formed substrate is degraded in normal metabolism and the assumption would seem quite probable, were it not for the fact that sometimes other enzymes are discovered in the intact cell which can act on the particular substrate, although in a different reaction. The substrate must, therefore, enter at least the periphery of the cell and the explanation of the phenomenon is still wanting.

It is possible that the inactive enzyme is more isolated in the cell than the active one, perhaps separated from the substrate by a membrane (cytoplasmic, mitochondrial, etc.), an inhibitor, or a surface charge. In this state exogenous substrate is unable to reach the enzyme and thus no reaction takes place. When the same substrate, on the other hand, is produced endogenously it may be inside of the supposed barrier.

A second possibility is that the enzyme is bound <u>in situ</u> in a relatively inactive state due to low energy bonding, for instance, it may be adsorbed to some interface such as

at the surface of a lipid or nucleic acid structure. Such a bound enzyme might be able to degrade endogenous substrate because, speculatively, the energy of phosphate bonds in the substrate might be transferred to allow desorption of the enzyme from the interface. Alternatively the partially active sites of the enzyme may require the substrate to be presented in a manner that is very unlikely to occur when it is exogenously supplied.

A third possibility is that the substrate can be used only in an "activated" form; for instance, by virtue of still being bound to the enzyme molecule that synthesized it, it may have a greater affinity for new enzyme sites. This possibility seems distinctly less likely, in view of the fact that cell extracts can carry out the enzymatic process without any additions. This makes necessary the postulation of a bound substrate activator, a concept not essentially different from either of the first two.

One of the enzyme systems in which the phenomenon of enzyme unmasking has been most frequently observed is that

involved in the oxidation[1] of citrate. This system involves two primary enzymes, aconitase and isocitric acid dehydrogenase, both of which are extractable from bacterial cells in appreciable amounts and generally independent of the ability of intact cells to oxidize citrate. Citric oxidase is used by the cell as part of the tricarboxylic acid cycle, but can frequently oxidize only endogenously formed citrate.

Escherichia coli and Brucella abortus display this property in a marked fashion. Since the functioning of the tricarboxylic acid cycle has been well established in the former and partially in the latter they were chosen for further study. Several enzymes that will split citrate anaerobically also have been demonstrated in E. coli: citric desmolase[18,19,20] citritase[99,100] and isocitritase[10,82,83]. These enzymes appear to function only to bypass the block preventing the entry of citrate directly into the tricarboxylic acid cycle in much the same way that surface enzymes enable certain yeasts to split sucrose to the more permeable monosaccharides[72]. Oxidation of the end

1. Throughout this thesis, the term "oxidation" is used to denote enzymatic reactions coupled to oxygen, the equivalent of "aerobic respiration". For convenience, the enzyme complexes mediating such a series of reactions we call "oxidases".

products of citrate splitting is not observed because of rate limitations, which are due to the small quantities of products produced per unit of time. This block in citrate oxidation can be removed by various treatments of the intact cells, such as disruption or drying. As these are non-specific treatments they demonstrate only the presence of the enzymes and do not suggest an explanation for the peculiarity of their behaviour.

The problem then is: If the enzymes for the oxidation of citrate are present, why is the intact cell unable to carry out this reaction on exogenously added citrate?

A working hypothesis to answer this problem postulated that because of barriers external to the active enzyme sites, exogenous citrate is excluded. These barriers are not at the outer surface of the cell and any treatment which either displaces them or destroys their integrity, enables the cell to oxidize citrate, providing the enzymes necessary are not also destroyed. These barriers may be in the nature of a membrane, an inhibitor, or the enzyme.

For purposes of investigation, two approaches were used: (1) Can the oxidation of citrate be brought about by altering

environmental conditions? (2) Does citrate penetrate an appreciable internal volume of the cell, and if so can this penetration be altered by altering the environmental conditions?

The first of these approaches was tried initially in the hope that it would be the more rewarding. Such things as pH, energy or ionic requirements, inducibility, etc. were studied, using as the rationale, in general, instances from published reports where similar variables influenced the rate of oxidation of organic compounds.

Since all attempts to induce citrate oxidation by any means other than complete or partial cell disruption were unsuccessful, the second approach was initiated in an attempt to get some indication of the degree of penetration of citrate. Variables similar to those used in the respiration studies were tried. As it was thought that the cell was not completely impermeable to citrate, a study of the characteristics of penetration should give some indication of the block to citrate oxidation, i.e. whether there is an actual membrane closing off a large part of the cell, or a

more subtle barrier at a mitochondrial or even an enzyme level.

HISTORICAL

1. Bacterial permeability

In the last decade a number of general reviews on permeability have appeared[12,21,49,64,71,94]. Although most studies have dealt with higher plant and animal cells, some have employed yeast or bacteria as test systems. The use of bacteria is an obvious one, although it has not been frequently exploited, due perhaps, to their size which makes direct observation difficult. Among the advantages to be gained from the use of bacteria in the study of permeability are ease of production, and greatly increased surface area per unit weight of cells. It would also be of considerable importance to both the physiologist and biochemist to know more about the actual process of penetration of metabolites, as well as other organic and inorganic compounds into bacterial cells.

From the limited studies that have been made, and by inference from metabolic studies, it generally has been concluded[34,51,56,65] that bacterial cells are fairly selective

in what they allow to penetrate their outer membranes. In this they appear to be similar to higher forms.

In contradistinction to this general picture of semi-permeability, Roberts et al.[70] in a recent monograph on biosynthesis in E. coli have concluded on the basis of "water space" measurements with a number of different compounds that "the protoplasm may be likened to a sponge, the cell membrane to a surrounding hair net unable to exclude the entrance or emergence of small molecules. Into this system water may diffuse freely and carry with it many of the dissolved substances which it contains. Thus, the nutrients of the environment diffuse into the reactive sites of the protoplasm. If they are metabolically acted upon they may become nondiffusible." If their conclusions are valid it will be necessary to revise the present concepts of permeability with respect to the bacterial cell considerably.

Recently[56] Mitchell and Moyle have taken issue with these conclusions. They report attempts to repeat the findings of Roberts et al. but have been unable to do so in either E. coli or Staphylococcus aureus. Similarly, Gerhardt

(unpublished results) has been unable to repeat the observations of Roberts et al., in so far as the amount of water space which they report (water space of 75%). Both Mitchell and Moyle, and Gerhardt obtain space values of about 40%. More recently moreover, Roberts et al. (personal communication) have been unable to repeat their original findings, and now report values of 40 to 50%. The reasons for this discrepancy are unknown, but are not thought to be due to methodology.

In the work of both Roberts et al.[70] and Mitchell and Moyle, the method of analysis of cell space is open to criticism. Roberts et al. used rather small quantities of cells and did not employ adequate controls on the intercellular space. Mitchell and Moyle, on the other hand, present their data in a confusing manner in that they report the volume per gram dry weight not penetrated by the substrate under study. When this value is converted to the volume actually penetrated and expressed as a percentage of the whole cell volume, space values of from 10 to 45% are obtained even if we accept the very high intercellular space value of 25% which they have used. Although these corrected

space values are smaller than the maximum values which Roberts et al. reported, they are large enough to be considered as good evidence for penetration and in contrast to Mitchell and Moyle's conclusions of general impermeability cannot logically be explained by adsorption. As a case in point, they are unable to explain the inability of glycine to stabilize protoplasts at 2°, at which temperature they have concluded that "intact cells are known to be impermeable to glycine". A recalculation of their data, however, indicates that approximately 40% of the cell is permeable to glycine at this temperature. This value seems large enough to explain the "profound difference" between intact cells and protoplasts; since glycine enters the intact cell it could not be expected to stabilize the protoplast.

As has been pointed out above, there is good evidence for the penetration of compounds by free diffusion across the cell membranes. There is also a considerable amount of evidence for a concentrating mechanism for certain ions and metabolites in the presence of an energy source. This condition was shown by Cowie, Roberts and Roberts[16] to occur in

the case of potassium penetration in E. coli. They concluded that the concentration of potassium in the cells could be attributed to the formation of non-diffusible potassium compounds during metabolism. In a later publication[68] they extended this observation to several other ions.

In a similar vein, Gale[31,32,33] has noted that lysine passes across the cell wall of Streptococcus faecalis, apparently by simple diffusion processes; the lysine is not lost when the cells are incubated in water. The addition of glucose and subsequent stimulation of metabolism, however, allows a limited amount of lysine to diffuse out of the cell. Attempts to explain this movement of substances have resulted in two theories: (1) The cell membranes are permeable to the substance concerned which is held by electrical or chemical combination on the side of greater concentration; (2) The cell membrane is impermeable to the substances concerned but they are able to migrate across the membrane in a different physical or chemical state from that in which they accumulate on one side. Supporting the first assumption, Gale was able to show that, under physiological conditions, lysine is

positively charged while the bacterial cell is negatively charged. This he felt would account for the diffusion of lysine into the cell. The glucose appears to have the effect of discharging the specific particle that is responsible for holding the lysine molecule within the cell. A further interesting observation is that lysine enters the cell at a maximum rate at pH 9.47, the isoelectric point of lysine, from which it was concluded that the lysine passes across the cell wall in an isoelectric state but accumulates within the cell in a cationic state.

As well as permeability due to diffusion across the cell membranes, Gale observed a second type of penetration occuring in S. faecalis. If these cells are grown on a medium with a low concentration of amino acids and then exposed to a solution of glutamic acid, no uptake of the glutamic acid is observed even after incubation at 37^{o} for a period of three hours, in contrast to the uptake observed with lysine under these conditions. If, however, glucose is added to the external medium, then glutamic acid is readily taken up by the cell until an equilibrium is established.

Arginine could also act as an energy source for the migration although it is less efficient on a mole per mole basis. In the presence of glucose the migration is inhibited by iodoacetate and sodium fluoride in concentrations inhibitory to glycolysis. These observations would tend to support the second theory, i.e. glucose supplies energy either for the transformation of the penetrating molecule, or for the actual passage itself.

In addition to the net penetration of materials noted above, there may be an exchange of substances back and forth across the surface membranes of a cell. Mitchell and Moyle[53,54,55], in studying the rate of phosphate exchange across the surface of S. aureus, have concluded that the exchange occurs by means of carrier groups in the cell surface which carry phosphate ions either way at a constant rate. This exchange can be inhibited by very small amounts of phenylmercuric chloride. Roberts and Roberts[68] have studied a similar exchange in E. coli but they have concluded, on the basis of their free diffusion theory, that the exchange takes plase between adsorption sites within the cell.

In yeast, Rothstein[72,73] has found evidence for the presence of a hexose transporting enzyme which acts at the surface of the cell in conjunction with sucrase. It is specific for glucose and is thought to be a species of hexokinase. Rothstein does not, however, feel that this enzyme system for the fermentation of glucose is present at the surface of the cell and therefore permeation is unnecessary. The difficulty with this view, however, is that he considers the "surface" to be that part of the cell remaining after dried cells are washed free of soluble protein. It seems probable that structures such as mitochondria and microsomes, which are known to contain a number of enzyme systems that may account for much of the observed enzyme activity, would not be removed by this treatment. Such structures should not be considered surface structures.

Recently several carrier systems have been reported which require the induced biosynthesis ("adaptation") of a specific transporting enzyme. Barret, Larson, and Kallio[6] first noted this adaptive response in Pseudomonas flourescens with respect to citrate oxidation. Williams and Wilson[97]

concluded, after studying the effect of succinate adaptation on succinate uptake in Azotobacter vinelandii, that adaptation did not increase succinate penetration in the absence of metabolism. They suggest that an active transport system may be present but offer no supporting evidence.

More recently, Monod[57] has reported the discovery of a transporting enzyme for galactose in yeast and E. coli, which can be induced by several galactose analogs. By the use of these analogs he was able to selectively induce a "permease" system without concomitantly inducing the galactosidase system. He was also able to show that, in the absence of the permease system, induction of the galactosidase system could not take place. This was presumably due to the necessity for a high concentration of substrate within the cell in order to obtain induction of the enzyme. Such concentration was dependent on the presence of a concentrating enzyme (permease). This mechanism appears to be of widespread occurrence in bacteria and yeast and seems to apply to a number of substrates (Dr. Harlyn O. Halvorson, personal communication).

Many workers have recognized the dependency of metabolic activity on the pH and concentration of the substrate. Recently Simon and Beevers[80,81] have analyzed and constructed a generalized plot of 90 different pH experiments, many in themselves incomplete, and have come to the conclusion that usually "the effect of pH on the degree of dissociation of the acid or base in the external medium is responsible for a large part to the effect of pH on activity, but it cannot be held that only the undissociated molecules are active". Erlandson et al.[25,26] have used these variables to investigate the rate of succinate penetration into Brucella abortus. Although their findings were complicated by indirect analyses, their results in general agree with those of Rothstein[72] as to the effect of pH on surface and internal enzymes.

2. The metabolism of citrate by Escherichia coli and Brucella abortus

The inability of E. coli to utilize citrate for growth has been recognized for many years. Koser[44,45] was among the first to recognize this phenomenon and employed it as a diagonostic tool for the separation of E. coli from Aerobacter aerogenes: He further noted[46] that this character

in E. coli was very stable, but recognized that there was some multiplication of organisms in the citrate broth employed. Some years later Parr[66] clearly demonstrated that E. coli strains fell into three groups: (1) Those unable to grow on citrate or to form mutant citrate oxidizing clones. (2) Those able to form citrate oxidizing clones. (3) Those able to use citrate as a sole source of carbon. Some years after this Zamenhof[101] used a mutable strain of E. coli to study factors affecting the time of appearance of mutants. Although the results of this study are inconclusive, they serve to confirm the citrate mutability of certain strains of E. coli.

Other workers[8,20,50,82,95,99,100] using strains which are not genetically different from the "natural" non-citrate oxidizing types, have studied the ability of E. coli to decompose citrate anaerobically and aerobically. They have concluded that this is effected by the involvement of "splitting" enzymes formed by E. coli when grown in a peptone or acetate medium with citrate present. Among the first studies on the citrate splitting enzymes were those of

Campbell *et al.*[10], who reported the presence of an enzyme, isocitritase, which splits citrate to glyoxylate and succinate in *Pseudomonas aeruginosa*. This enzyme was later[83] found to require Mg^{++} and a sulfhydryl activator such as cysteine or glutathione for full activity and was not active on citrate or *cis*-aconitate in the absence of aconitase. Isocitritase occurs in a number of organisms including *E. coli*[82]. In *E. coli*, however, it is formed only aerobically when acetate is used as an additional carbon source in contrast to its anaerobic formation in other organisms.

A second enzyme, citridesmolase, has been reported by Dagley and Dawes[18,19,20] which likewise splits citrate to a C_2 and a C_4 fragment. Here the products are acetate and oxalacetate. This enzyme is formed adaptively in cells grown anaerobically in the presence of glucose or peptone. In the intact cell the oxalacetate is rapidly decarboxylated to pyruvic acid so that, unless the latter reaction is supressed by appropriate inhibitors, oxalacetate can not be demonstrated. More recently a similar enzyme, citritase, has been isolated and characterized by Wheat and Ajl[99,100].

It is formed by cells grown anaerobically in the presence of glucose and requires Mg^{++} but not CoA for its activity. It is probably identical to the citridesmolase of Dagley and Dawes[20].

The third and predominant pathway of citric acid breakdown is the stepwise process of the tricarboxylic acid cycle. All component enzymes can be isolated in significant quantities from normal cells, and it has been frequently shown that this pathway is operative in E. coli. Citrate itself is not acted on when added exogenously, but when oxalacetate and acetate as the fluoride salt are added, large quantities of fluorocitrate pile up[63,70]. In the absence of fluoride, slight traces of citrate are found. This is taken to indicate that the cells are capable of utilizing endogenously formed citrate.

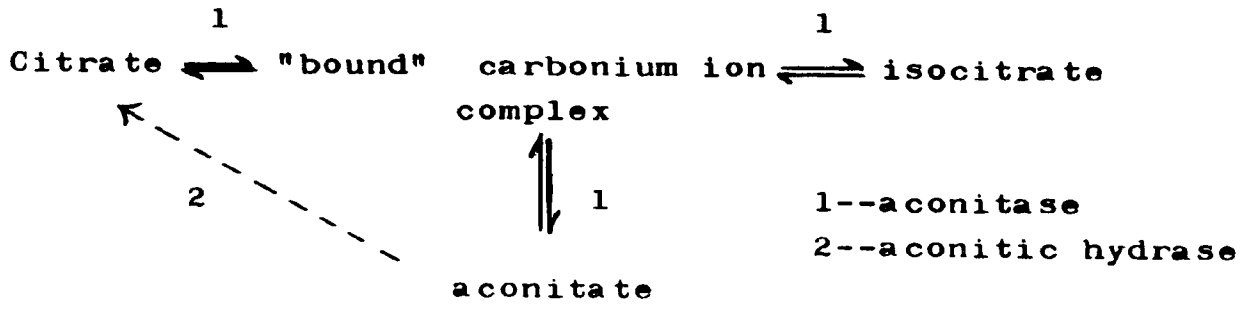
Stern and Ochoa[87] have shown that acetate and oxalacetate will condense to form citrate in the presence of extracts of E. coli and have isolated the condensing enzyme which requires CoA in appreciable quantities. Thus one might expect a fourth method of citrate breakdown, the

reverse of the condensation, which would require CoA. This has not yet been demonstrated.

Since the first two pathways are active anaerobically, the only aerobic pathway, yet described, which would be possible is via the tricarboxylic acid cycle. Possibly the splitting enzymes can form enough end products to enable measurable oxidation to take place, but this is unlikely under most experimental conditions. It has furthermore been shown that E. coli readily oxidizes citrate if the cells are mechanically disrupted, dried, or lypholized[1,48]. In dried cells Ajl et al.[48] were able to show by means of radio-isotopes that the tricarboxylic acid cycle was the only pathway of citrate degradation, although they carefully looked for a C_2-C_4 split.

The enzymes necessary for aerobic citric acid oxidation, therefore, are aconitase and isocitric acid dehydrogenase. Aconitase requires Fe^{++} and cysteine, although other sulfhydryl activators can be used[23,58,59]. Speyer and Dickman[85] have recently presented good evidence for the non-involvement of cis-aconitic acid in the degradation of

citrate to isocitrate, and have postulated a "bound" carbonium ion complex as an intermediate, which in the presence of aconitase can form citrate, aconitate, or isocitrate at equal rates:



Neilson[6] has reported a new enzyme in Aspergillus niger which is able to convert aconitate irreversibly to citrate. Although the occurrence in other organisms of this enzyme, which she calls aconitic hydrase, is not reported, it would seem to readily fit into the scheme of Speyer and Dickman shown above. Isocitric dehydrogenase degrades isocitrate to oxalosuccinate by a TPN linked reaction which requires one mole of O_2 for every mole of isocitrate.

In B. abortus there is very little known about the method of citrate degradation. Gerhardt et al.[35] found a

high rate of oxidation in intact cells in the presence of DL-isocitrate but no significant increase over the endogenous with citrate. Although no explanation was provided, (in a subsequent report[34] isocitrate was not studied further) this reported oxidation may possibly be due to contamination with succinate (see Results, below). It was reported, however, that ruptured cells were able to oxidize citrate. Altenburn and Housewright[4] studied the oxidation of citrate by large masses of cells and came to the conclusion that it was oxidized by intact cells at a low rate. On the basis of $QO_2(N)$ values, however, this rate increase is very questionable. They showed further that in the presence of fluoro-acetate citrate accumulated, indicating that the tricarboxylic acid cycle probably functioned normally. It was suggested that the low rate of citrate oxidation by intact cells was due to the rate of penetration of the substrate. To date no citrate dissimilating enzymes have been reported for B. abortus, but a search for them may be rewarding.

MATERIALS AND METHODS

1. Cultures and Cell Preparations

(a) Escherichia coli

Strain B of E. coli was obtained from Dr. D. B. Cowie of the Department of Terrestial Magnetism of the Carnegie Institution of Washington. Stocks were maintained on trypticase-soy agar and stored in the refrigerator. Cells were grown on the M-9 medium of Anderson[3]: 0.2% NH_4Cl, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% NaCl, 0.01% $MgSO_4$, 0.8% glucose. Either a fifteen liter carboy or a thirty liter stainless steel fermentor were employed. Enough antifoam (Dow Corning AF) was added to control foaming. In both cases the inoculum was 700 ml of a 24 hour shaken culture grown on M-9 medium at 30^{o}. Aeration in the carboys was the maximum obtainable with two stone spargers. Cells were harvested when the cultures reached a maximum turbidity, which varied from 8 to 24 hours, depending on the temperature employed. This level of turbidity was taken to indicate the end of the log phase of growth.

In the stainless steel fermentor, cells were mechanically agitated and aerated under 10 pounds/sq in air pressure. The temperature did not vary usually from the desired 34°. Cells were harvested at 10 hours by means of a Sharples super centrifuge. Cell yields varied from about six grams wet weight per liter in the case of the carboys, to 20 grams per liter in the fermentor. Due to the much greater effeciency of the latter, it replaced the carboy as a culture vessel.

As treatment of the cells in each batch subsequent to harvesting varied with the experimental conditions, it will be given with the description of the particular experiment. In all cases careful checks of the cultures for contaminants were made. Three criteria were used: gram stain, morphological observation of unstained cells, and streaking on Simmons citrate agar. The only contaminant ever found was a pseudomonad which was able to grow on citrate as a sole source of carbon. The presence of this organism could profoundly alter the results obtained in citrate uptake experiments. If the cells did not grow on citrate agar, no

further concern over possible contamination was considered necessary.

(b) Brucella abortus

Strain A-19 of Brucella abortus (smooth) was used. Procedures for its storage and preparation have been described by Erlandson[26]. Cells were grown on modified tryptose agar (2% Bacto tryptose, 0.1% glucose, 0.5% NaCl, 0.1 μg/ml thiamine hydrochloride, and 2% Bacto agar) at 37° for 24 hours. A slight modification from the procedure described in detail by Erlandson[26] warrants description here. Stock cultures, which were stored at -40°, were thawed and the cells were innoculated onto agar slants, which had been stored to give a dry surface. The cells which grew on these slants were scraped off with a large wire loop. A pellet of cells about the size of a small pea was used as an innoculum for a penicillin culture flask (a wide mouthed Roux type flask) in which the agar had been previously aged to insure dryness of surface. This procedure increased the cell yield many times. Due to the dry surface on the culture flasks it was possible to detect easily and remove colonies of contaminants.

Cells were harvested by washing the agar with buffered saline (0.067 M phosphate buffer plus 0.9% NaCl). They were then washed three or four times in buffered saline before use.

For large masses of cells, Albimi broth cultures were employed and harvested by means of the Servall SS-1 centrifuge. As the use of broth cultures is a somewhat hazardous procedure, several other methods were tried. Two methods were successful in increasing the yield although they were not quantitatively studied. If a thin layer of agar medium was hardened on the entire surface of a five gallon carboy, good growth could be obtained in 24 hours. The cells were easily removed but the agar had a tendency to dislodge. This method had the advantage of being simple and free from aerosols, but the handling of such a large container was somewhat dangerous. In the other method a flask half-full of hardened agar medium was used. Over this was poured a small volume of broth. This was inoculated and incubated on a shaker. Cell concentration appeared to be increased about tenfold.

2. Respiratory analyses

(a) Brucella abortus

Measurement of the respiration of resting cells of B. abortus was carried out by the use of conventional manometric techniques described by Umbreit, Burris and Stauffer[93]. A standard Warburg, constant-volume microrespirometer was employed. Flasks were shaken at the rate of 118 complete strokes per minute through a distance of 5cm. Each flask contained 0.2 ml of 20% KOH in the center well, 1.0 ml of cells in a concentration of about 400 µg of nitrogen per ml, 1.5 ml of appropriate buffer in the main flask, and 0.5 ml of substrate in the side arm. Flasks were equilibrated for 20 min before the manometers were set and the substrates added. For rate studies twelve readings were taken at 10 min intervals. Rates are experssed as microliters of oxygen uptake per hour per milligram of cellular nitrogen ($QO_2(N)$) and represent the average of duplicate determinations. Rate data were computed from the linear portion of the curve. It should be pointed out that $QO_2(N)$ values vary somewhat from experiment to experiment due to the use of different batches

of cells. This variation is most apparent in the endogenous respiration (see tables 2 and 3).

(b) Escherichia coli

The same procedures were used with E. coli as with B. abortus, except that the cell quantities varied. Rates with E. coli are expressed as microliters of oxygen uptake per hour per mg dry weight of cells (QO_2).

With both B. abortus and E. coli, rates for disrupted cell preparations are expressed on the basis of nitrogen or dry weight in the original cell mass and not on these values for the extract.

(c) Nitrogen analysis and cell dry weight

The nitrogen content of the cell suspensions was determined by the method of Umbreit, Burris, and Stauffer[93] as modified by Erlandson[26].

Cell dry weight was determined by pipetting 1.0 ml of the cell suspension into each of three tared aluminum weighing dishes and drying to a constant weight in a 110° oven.

(d) Preparation of substrates

For all respiratory analyses, substrates were dissolved in 0.067 M phosphate buffer at the desired pH and concentration. pH adjustment, if necessary, was made with KOH or phosphoric acid. All solutions were freshly prepared before use from reagent grade chemicals.

3. Substrate Analyses

(a) Citrate analysis

A number of citric acid analyses with varying degrees of accuracy and sensitivity have been developed. For the most part these analyses are usable only with very small quantities of citric acid. The precision of one of the best of them, that of Natelson et al.[60] is ± 5% through a 20 µg range. This error becomes important if the original sample is high in citric acid content and so requires dilution. Cartier and Pin[11], however, have described a method useful from 100 to 1200 µg of citrate with but a small degree of variation (± 5 µg over the whole range). This method has the disadvantage of having a photo-sensitive final color formation which is influenced by the reagents used. Thus it

does not always produce a straight line for a standard curve.

The method finally evolved was a modified combination of the methods of Natelson et al. and Cartier and Pin. The initial steps were essentially those of the latter with the exception of the double washing which was reduced to one. Color was developed with Natelson's thiourea reagent, and measured immediately in a photocolorimeter using a 430 mμ filter. As the slope and y-intercept varied somewhat with each analysis, a three point standard curve was desirable for each analysis, although two points were usually sufficient. Within the range tested (50 to 1500 μg of citric acid), variation was found to be only about ± 5 μg among replicate samples.

Reagents: 10% trichloroacetic acid; concentrated sulfuric acid; potassium permanganate, 1.5N (47.1 gm/1); bromide-bromate solution, 2 N (42.88 gm sodium bromide + 12.58 gm of sodium bromate dissolved in 250 ml of water); hydrazine sulfate, 10 N (65 gm are placed in 200 ml of water, alkali added until hydrazine dissolves and the solution made

up to 250 ml); saturated bromine water; n-heptane; thiourea solution pH 9.2 (2 gm borax dissolved in 100 ml of 4% thiourea); citric acid standard containing 1.00 mg of citric acid per ml.

Procedure: Soluble protein was removed from the sample by adding an equal volume of 10% trichloracetic acid. After centrifugation a measured volume of the supernatant fluid was placed in a glass stoppered vessel. (50 ml volumetric flasks were found to be satisfactory for this.) This sample, which should contain between 50 and 1500 μg of citric acid, was then made up to 5.0 ml with 10% trichloracetic acid, 1.5 ml of concentrated sulfuric acid and 1.0 ml of saturated bromine water was added and the flask placed unstoppered in a boiling water bath for 10 min. It was then cooled to about 4^{o} and 1.0 ml of bromide-bromate solution was rapidly added by means of a syringe and the flask vigorously shaken. One ml of 1.5 N potassium permanganate solution was added and the flask stoppered and allowed to stand for 30 min at room temperature. At the end of this time the solution was cooled to less than 4^{o} and decolorized by the dropwise addition of hydrazine sulfate solution (about 0.35 ml) with

vigorous shaking. The amount of decolorizing agent varied with each sample and so cannot be accurately prescribed. To this clear solution, 15 ml of n-heptane was added and the mixture shaken for 5 min on a reciprocal shaker. The aqueous layer was removed by aspiration and 10 ml of distilled water added to the heptane layer. This mixture was shaken 3 min and three ml of the top heptane layer removed and placed in a large test tube to which 6.0 ml of thiourea solution were added. The tubes were capped and shaken for 5 min, after which the yellow aqueous layer was removed and its optical density determined in a Coleman model 9 Nephocolorimeter using 12 x 75 mm round cuvettes and a 430 mμ filter.

For some of the earlier work reported, citrate was estimated using the method of Saffran and Denstead[75]. This method was abandoned when it was found that some component of the cellular protein interfered with the reaction.

(b) Pyruvate Analysis

Pyruvate and total alpha-keto acids were determined colorimetrically by the method of Friedemann and Haugen[30].

(c) Inulin Determination

Inulin was determined by a gravimetric procedure. Due to the nature of the compound, however, drying the samples by heat did not give reproducible results. When the samples were dried first *in vacuo* over calcium chloride to remove the excess water and then over phosphorous pentoxide until a constant weight was reached, very reproducible results were obtained.

4. Space Determinations

(a) General Considerations

If one considers the bacterial cell as a fluid, gel-like structure contained in a rigid, interwoven framework, then it will be realized that when this bacterium is immersed in a liquid which can penetrate part of it, the volume of liquid displaced by the cell will be somewhat less than the volume displaced by a solid sphere of equal dimensions which the suspending liquid is not able to penetrate. The apparent decrease in the volume of the displaced liquid will be in direct proportion to the amount of fluid which penetrates the cell. If this fluid contains dissolved substances, they will also enter the cell along with the suspending liquid, if there are no

barriers to prevent their passage. In general the suspending fluid is water and since living cells are usually saturated with water there is no net penetration when a cell is placed in it, although there is a diffusional exchange in that the cell allows the water to pass in and out freely. This saturation is not true for most substrates, on the other hand, and if they are able to diffuse into the cell, they will assume a concentration equal to that of the external medium. This results in a decrease in concentration in the external medium which is directly proportional to the amount of penetration of the substrate into the cell. Thus, by measuring the change in concentration after adding a known amount of cells to a known substrate concentration, we can determine the portion of the cell which has been penetrated by the substrate, that is, the "substrate space". In other words, the substrate space of a cell is that fraction of the total cell volume which acts only as a diluent for the substrate. For example, a citrate space of 0.75 indicates that 75% of the total cellular volume is occupied by citrate equimolar to that outside of the cell.

It should be pointed out, moreover, that substrate space does not imply either adsorption or internal concentration of the substrate. It indicates only an equilibration of the internal with the external medium, in which there is free exchange of molecules as in any diffusional system. Unfortunately, it is impossible by means of the techniques employed to distinguish whether the space found in cells is due to a purely diffusional phenomenon, to adsorption, or to concentration of the substrate in a small portion of the cell either by an enzyme or by some physical phenomenon such as the Donnan equilibrium. Experiments can be designed, however to clarify the nature of the space for any given substrate and bacterium. For instance, an adsorptive mechanism ought to reach saturation with increase of external substrate concentration as should a concentrating mechanism (such as a "pool") within the cell. On the other hand if enzyme activity is involved, the space ought to be markedly affected by temperature and pH.

It can be seen that if a substrate is excluded entirely from a cell by an impermeable membrane at the outer surface,

the resulting substrate space will be zero. On the other hand, if the substrate penetrates only the cell wall, a substrate space of from 0.10 to 0.15 may be expected, assuming the wall to be 10 to 15% of the whole cell. If the substrate freely penetrates the interior of the cell and equilibrates with the cell water (= 75% of the total wet weight) one might expect a space of 0.75. A space higher than this would indicate some concentrating mechanism such as adsorption or metabolism. Theoretically a water droplet, being entirely available for dilution, would have a space of 1.00. Thus, space values approaching or greater than one have little meaning in terms of space in the cell but may have some meaning in terms of concentrating mechanisms, mentioned above.

When large masses of cells are packed tightly together, their weight can be assumed to be approximately equal to their volume (specific gravity = 1.1). However, a wet pack of cells will contain at least a little water in the interstices between the cells which will be maximal for non-compressible spheres. Conway and Downey[15] have pointed out

that with such non-compressible spheres the intercellular space is at a theoretical maximum of 0.477 independent of the diameter of the sphere. If, on the other hand, the spheres are "deformed to parallelopipeds, with 60 and 120° angles in the faces, one has an arrangement of closest packing and the interspace as a fraction of total volume is....0.26." As the spherical arrangement is further deformed the intercellular space continues to decrease. As this deformation is easily effected by increasing the speed and time of centrifugation, these variables ought to be held constant, if a constant intercellular space is to be obtained.

The intercellular space can be measured by the amount of dilution of a substrate which does not penetrate the cell when mixed with the packed cells. These non-penetrating substances are chosen by virtue of their molecular weight and chemical inertness. For E. coli, inulin was found to be quite satisfactory. Other substances, however, could be used, such as high molecular weight dextran and protein molecules. Their usefulness can only be determined by actual trials.

(b) Calculations

If a suspension of cells is packed to a constant weight (Wp) it will have an intercellular space (Sin) which is also constant. This space can be determined by adding to the pack of cells a given volume (Vs) of an impermeable substrate (here, inulin), at a known concentration (Co). On suspension of the cells in this solution, a certain amount of dilution of the substrate will occur due to the intersitial water, resulting in a total solution volume of Vs + Sin. Wp (where Sin = the interstitial volume per gram of cells) at a final concentration Cf.

Thus: initial substrate = final substrate

$$\text{or } Co \cdot Vs = Cf(Vs + Sin \cdot Wp) \qquad (1)$$

therefore,

$$\frac{Co \cdot Vs}{Cf} = Vs + Sin \cdot Wp \qquad (2)$$

or,

$$\frac{Co \cdot Vs}{Cf} - Vs = Sin \cdot Wp \qquad (3)$$

which can be rewritten as,

$$Vs\left(\frac{Co}{Cf} - 1\right) = Sin \cdot Wp \qquad (4)$$

This equation can be rewritten as an expression of the intercellular space,

$$\frac{Vs}{Wp}\left(\frac{Co}{Cf} - 1\right) = Sin \qquad (5),$$

from which it is readily apparent that Sin is an expression of ml of intercellular volume per gram of cells.

On the other hand if the substrate is one which penetrates the cells, the final concentration of substrate (Cf) will be decreased in proportion to the amount of substrate penetrating the cells as well as the intercellular volume. This substrate space (Ssub), which also may be determined by equation 5, will be equal to the sum of the intercellular space and the space of the cell which is penetrated. Therefore the actual cell space penetrated (R) will be equal to the total pack space (Ssub) less the interstitial space (Sin). However since the total pack weight as measured constitutes the total cell weight plus the interstitial space, and Ssub is based on a pack weight of one, the corrected cell pack weight will be greater than one because there is more cell material per unit weight than in the same weight of cells plus interstitial space. This can be corrected for by multiplying the corrected space Ssub-Sin, by $\frac{1}{1-Sin}$.

Therefore, $$R = \frac{Ssub\text{-}Sin}{1\text{-}Sin} \qquad (6)$$

When Sin is small, 1-Sin approaches one, therefore the equation can be simplified to

$$R = Ssub\text{-}Sin \qquad (7).$$

(c) Centrifugation and Resuspension

The longer a cell mass is centrifuged, the tighter it will become packed until a minimum is reached in which either the cell will not compress further or the intercellular space becomes zero. Initially the packing will be rapid, after which the rate will decelerate as the cells offer more and more resistance to compression. Thus, for purposes of obtaining a consistent cell pack with a constant intercellular space, it was found necessary to run an experimental curve on the rate of packing. As is shown in Figure 8 a consistent pack can be obtained by centrifuging the cells for 20 min at 20,000 G. In all experiments reported here, this time and speed were used for centrifugation unless otherwise indicated.

Once the cells were packed the supernate was quickly poured off and the sides of the tube were wiped free of

water. These dried, packed tubes of cells were then weighed to the nearest 0.1 g and placed in a 0^{o} water bath. An accurately measured amount of buffer, saline, or other diluent was then added and the cell pack was carefully resuspended. The amount of liquid added depended on the weight of the cells, since it was always desirable to keep the Vs/Wp ratio less than one. This tends to minimize the errors in estimating the Cf (see above). In the case of intercellular space determinations, 10 ml of a 3% solution always was added directly regardless of the cell weight.

When the cells were thoroughly resuspended and equilibrated at 0^{o}, a measured volume of substrate was added. This sequential addition of diluent and then substrate allowed rapid and reproducible diffusion throughout the cell mass. The mixture was then incubated at the desired temperature for 10 min, (or for varying periods in rate determinations) quickly transferred to the centrifuge, and repacked for 20 min at 20,000 G. Immediately after centrifugation the supernatant was decanted and a measured volume pipetted into an equal quantity of 10% trichloroacetic acid.

This deproteinization was necessary because generally a considerable amount of cellular protein was present and may have contained citrate-destroying enzymes. For intercellular space determinations, 4.0 ml of supernate was pipetted into each of two tared weighing dishes for gravimetric analysis. For substrate space determinations, the substrate-trichloroacetic acid solution was appropriately diluted and analyzed.

(d) Washing Procedures

If the amount of substrate that was capable of being washed from the cell pack was to be determined, the cells were packed for 20 min so that the intercellular space could be accurately corrected for. The pack surface was then quickly rinsed several times with water and the sides of the tube were swabbed dry. A measured volume of diluent was then added and the cells were resuspended and allowed to equilibrate for 10 min. The mass was again centrifuged and the procedure repeated as many times as desired. If there was free diffusion into the cells, one would expect free diffusion out, so that on the basis of the amount of

substrate lost in the supernatant analysis, it is possible to calculate how much substrate should be recovered in the wash by the formula,

$$\text{Substrate expected in the wash (E)} = \frac{V'}{V} \times G \quad (8),$$

where V' is the volume of added wash water, V = V' plus the volume of the cells and G is the total weight of substrate in the pack inferred from analysis of the supernatant. Percentage recovery can be determined from the formula,

$$\% \text{ substrate recovery} = \frac{\text{substrate found}}{\text{substrate expected}} \times 100 \quad (9).$$

Deviation from 100% recovery can be explained on the basis of strong adsorption, metabolism, or inaccuracy in analysis.

5. Cell Treatment Procedures

(a) Solvent Extraction

Cells that were to be solvent extracted were suspended in 30 ml of water and 2.0 ml of the solvent was added. The misture was placed on a shaker and shaken for 5 min at room temperature. Cells were used without washing. Butanol, chloroform and toluene were used in this manner.

(b) Mechanical Disruption

Two methods of mechanical disruption were employed,

sonic oscillation and grinding. For disruption by sonic oscillation, about 30 ml of approximately a 50% cell suspension was placed in the cup of a 10 kc Raytheon sonic oscillator, and treated until breakage approached 100% as determined microscopically. This took about 20 min. Unbroken cells and large cell particles were removed by centrifugation and the supernatant used as the cell extract. Grinding was accomplished by combining the cell suspension with an equal volume of "Superbrite" glass beads (Minnesota Mining and Mfg. Co., pavement marking beads, grade 10, average size 0.2 mm, sieve size 60/80) and mixing in a Waring blendor for 15 to 30 min[47]. The extract was obtained by centrifugation as above.

(c) Dried Cell Preparations

Dried cells were prepared according to the method of Lara and Stokes[48]. Thin layers of a heavy suspension of washed cells were spread on petri dishes and placed in a large desiccator over phosphorous pentoxide, in vacuo. These dried cells were then stored at 4^{o} in the refrigerator.

(d) Heat activation

Heavy suspensions of cells containing about 100 mg dry weight per ml were placed either in small sample vials or in the bottom of a Warburg flask. They were "activated" by immersing the container in a rapidly boiling water bath for the appropriate time. Reactions were stopped by immediately immersing the heated cells in 34° water bath. The volume and concentration of the cells are of importance and must be controlled carefully if the experiment is to be repeated.

6. Preparation of Cell Particles

A heavy suspension of E. coli cells was treated in the sonic oscillator for about one hour, the resulting suspension was diluted with water and centrifuged at low speed (5000 G) for 10 min in the Servall SS-1 centrifuge to remove the intact cells. The supernatant was then centrifuged at 10,000 G for 20 min. This pellet was resuspended in 0.9% NaCl and centrifuged at alternately low and high speeds several more times. The resulting high speed pellet was used as the cell particulate

fraction. It contained what appeared to be many cell walls as well as many small spherical bodies of unknown character, which may have been rolled up cell walls or membranes, or possibly some interior particles such as mitochondria.

7. Conditions for the selection of mutants

Heavy suspensions of *E. coli* were spread on a series of Simmons citrate agar plates and observed after several days for the presence of typical citrate-positive colonies. A large number of minute yellow colonies developed which on repeated transfer did not show any increased ability to grow in the presence of citrate as the sole source of carbon.

Because of the possibility that the ability of *E. coli* to mutate to a citrate oxidizing form was dependent on some energy source, ordinary Koser citrate broth was supplemented with either 0.1% acetate, aspartate, succinate, glucose, glutamate, or peptone. Cultures in these broths were analyzed for citrate content after several days incubation at 37^{o} and streaked on normal Simmons citrate agar to detect possible mutants.

RESULTS

1. The Respiration of Brucella abortus and Escherichia coli in the presence of citrate

A. The respiration of B. abortus, strain 19

1. The effect of addition of an energy source

It has been pointed out in an earlier section that a cell often requires an exogenous source of energy in order to allow penetration of a substrate. This energy may be required for the transfer of the substrate through a permeability barrier[22,33] or may be employed in the synthesis of a transporting enzyme. Also, studies with growing cultures of E. coli, which are able to metabolize citrate in the presence of other energy sources[8,50,52,91,95], suggested that perhaps resting B. abortus cells did not oxidize citrate because the process required a certain amount of exogenously added energy.

Thus the effect of various energy sources on the oxygen uptake of resting B. abortus in the presence of citrate (0.0033M) was studied first. As is shown in Table 1, neither 0.0033 M succinate 0.03 M, 0.0033 M nor 0.00033 M glucose effected any significant difference in

TABLE 1

THE EFFECT OF ENERGY OR NITROGEN SOURCE ON THE OXIDATION OF 0.0033 M CITRATE BY B. ABORTUS

Compound added	Molarity	pH	$QO_2(N)$[1] with Citrate	$QO_2(N)$[1] without Citrate
Succinate	0.0033	6.8	370	407
Glucose	0.033	6.8	478	392
	0.0033	6.8	448	431
	0.0033	5.5	526	550
	0.00033	6.8	240	237
Glutamate	0.000033	5.5	157	151
NH_4Cl	0.0033	5.5	157	151
None	-	6.8	133	133
	-	5.5	182	171

[1] A variation of $\leq \pm$ 20 is not considered significant

the rate of oxygen uptake by B. abortus in the presence or absence of citrate, at either pH 6.8 or 5.5. The lowest concentration of glucose was exhausted before the end of the experiment, but the deceleration in the rate of its oxidation was identical in both the presence and absence of citrate.

The possible requirement of the cells for a nitrogen source for the oxidation of citrate was also investigated. Neither 0.000033 M glutamate nor 0.0033 M NH_4Cl caused any significant change in the rate of oxygen uptake in the presence of citrate, as in shown in Table 1.

2. The oxidation of other tricarboxylic acids

In view of the fact that aconitase will act on any of the three tricarboxylic acids (isocitrate, aconitate, or citrate) it was postulated that perhaps all three together were required to spark the reaction. Accordingly solutions of the three compounds were mixed so that the resulting molar concentration for each acid in the mixture was 0.0011. Separate solutions (0.0033 M) of each acid were used as controls. The results of this experiment

are given in Table 2. There was no significant increase in oxygen uptake in the presence of the three tricarboxylic acids over that accounted for by the presence of DL-isocitric acid. The controls showed that in the presence of isocitric acid there was a considerable increase in the rate of oxidation, especially at pH 5.5, whereas aconitate and citrate caused no stimulation over the normal endogenous respiration even at the low pH.

3. Oxygen uptake in the presence of isocitric lactone

The preceeding experiment suggested that perhaps the citrate oxidizing block was in the conversion of the citrate to isocitrate. It is also possible that as a less polar lactone, the isocitrate molecule could more easily penetrate the cell. Thus a series of experiments was undertaken to study the oxidation of isocitric acid lactone.

As is shown in Table 2 the rate of oxidation of isocitric acid lactone is about three times as great at pH 5.5 as at 6.8. A unique feature of this high rate of oxidation at pH 5.5, however, was the deceleration of

TABLE 2

OXYGEN UPTAKE OF *B. ABORTUS* IN THE PRESENCE OF CITRATE, *CIS*-ACONITATE AND DL-ISOCITRIC ACID LACTONE

Substrate	Molarity	pH	QO_2 (N)
Citrate	0.0033	6.8	92
	0.0033	5.5	102
Cis-aconitate	0.0033	6.8	117
	0.0033	5.5	108
DL-isocitric acid lactone	0.0033	6.8	226
	0.0033	5.5	635
Sodium isocitrate	0.0033	5.5	189
Citrate, *cis*-aconitate, isocitric acid lactone	0.0033[1]	6.8	141
None	-	6.8	112
	-	5.5	90

[1] Total molarity. Molarity of each organic acid is 0.0011.

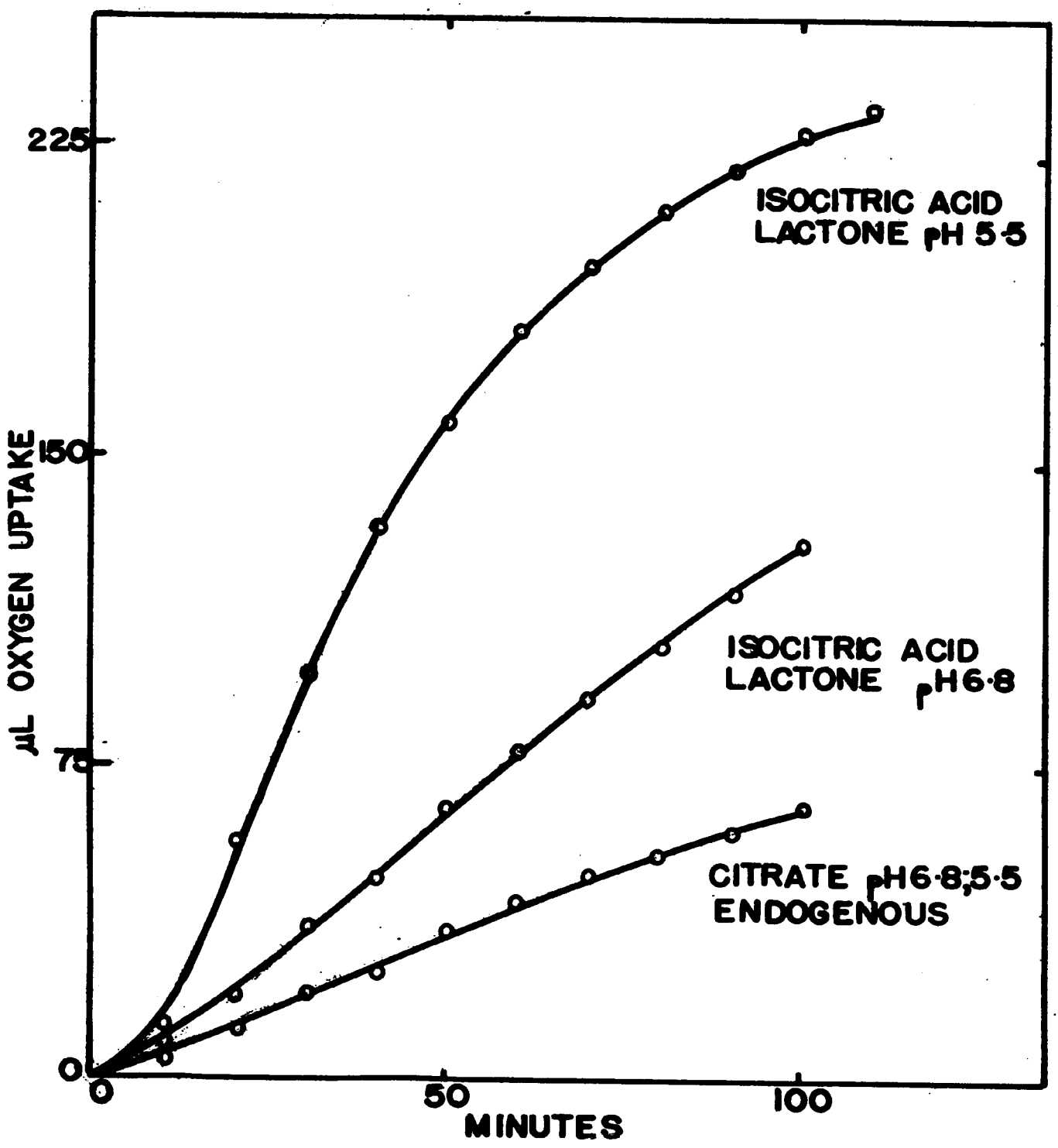


Figure 1. The pH response of B. abortus to isocitric acid lactone and citric acid. Substrate concentration 0.0033 M.

oxygen uptake until the rate was equal to that of the endogenous. This is shown in Figure 1. If the isocitrate is not degraded past oxalosuccinate or alpha-ketoglutarate, then it would not be expected that more than 224 µl of oxygen would be taken up for the complete oxidation of the 10 millimoles of the substrate added in the experiment. The fact that the "break" in the curve occurs after about 200 µl of oxygen uptake is suggestive of this possibility.

The lactone structure of the isocitric acid used suggested that this ring might render the molecule more readily available to the cell. Therefore, in order to convert the lactone more completely to the acid form, a solution was hydrolyzed for one-half an hour at pH 8.5 in a boiling water bath. Figure 2 shows that the rate of oxygen uptake after this treatment did not differ from that found with the unhydrolyzed substrate and suggests that the isocitric acid molecule alone was responsible for the oxygen uptake. It is also shown in Figure 2 that the deceleration of the oxygen uptake can be reversed by the addition of fresh substrate, indicating that the amount of substrate is limiting.

However, the foregoing evidence for the oxidation of isocitrate also could be interpreted as being due to the presence of a small amount of a contaminant. As is shown in Figure 2, isocitrate at a concentration of 0.00033 M produced no stimulation of oxygen uptake, although on the basis of the amount of substrate present, one would expect about 20 μl more oxygen uptake than in the endogenous control. The use of isocitrate from a different source (Nutritional Biochemical Company) gave a low rate of oxidation ($QO_2(N)$ of 189) as compared with isocitrate obtained from the California Foundation for Biochemical Research ($QO_2(N)$ of 407), which was used for most of these studies. The similarity between the pH response of isocitrate and succinate (at pH 5.5 the $QO_2(N)$ of succinate is about 650, and at 6.8 about 400) together with the fact that the isocitrate is synthesized from succinate is consistant with the possibility that the isocitrate used was contaminated with succinate. A recent report from the California Foundation for Biochemical Research indicates that at least one lot of

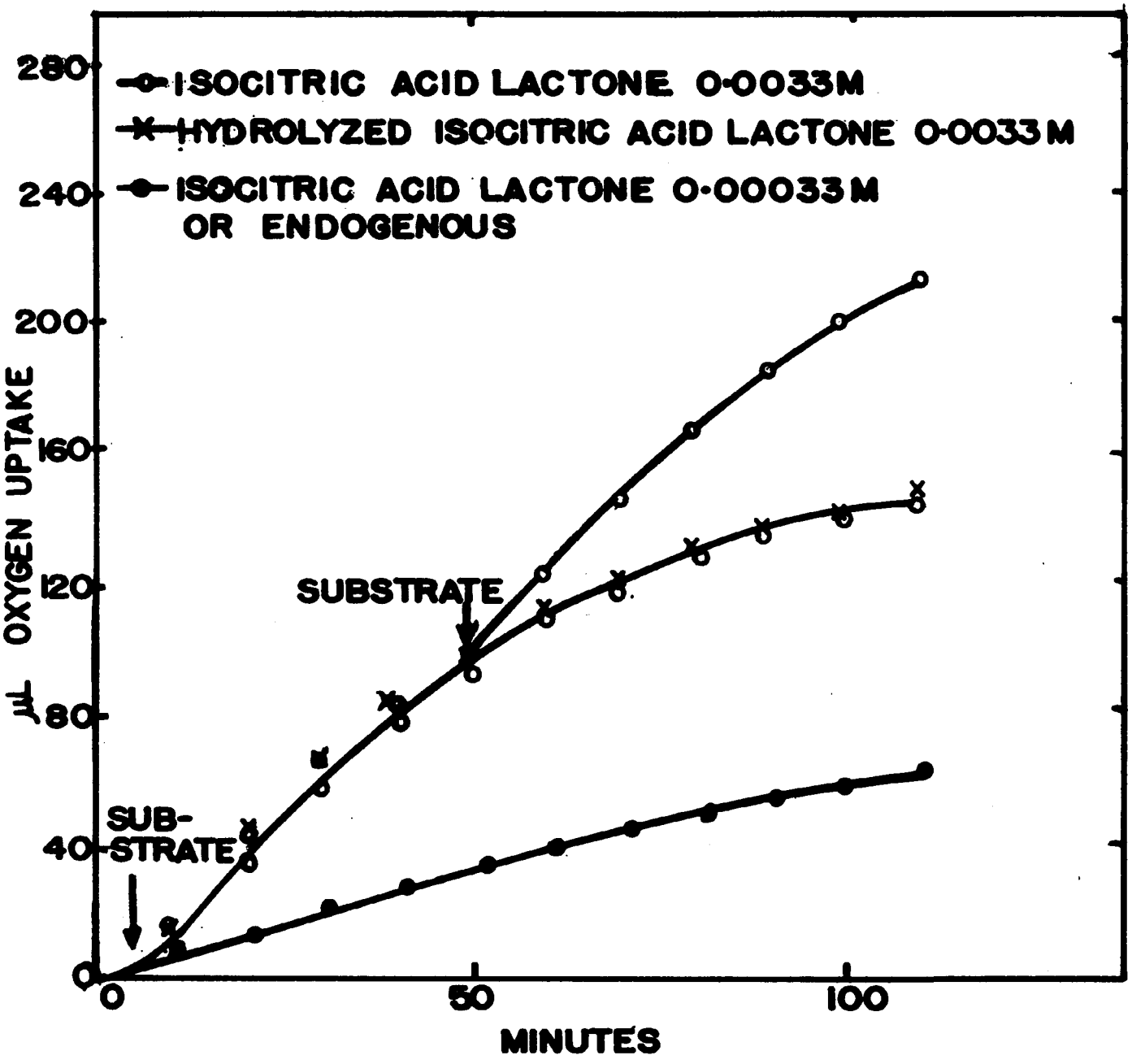


Figure 2. The effect of hydrolysis on the oxidation of isocitric acid lactone by B. abortus. pH 6.8, molarity of substrate 0.0033.

isocitric acid lactone was contaminated with about 30% succinate, but the company has no information on the lot employed in these experiments (III-13).

It is also possible that isocitric acid is oxidized only as the D or L form: the break in the curve representing the exhaustion of the isomer used.

4. The effect of growing cells in the presence of citrate

A number of workers have shown that when E. coli is grown in the presence of citrate that either citrate utilizing strains are selected[66,77,101] or that the cells respond adaptively to citrate[6,37]. Altenburn and Housewright[4] and Gerhardt (personal communication) however, were unable to induce adaptation to citrate oxidation, when the cells were grown in the presence of citrate. In order to investigate this possibility further 0.1% citrate was added to the usual culture medium. The cells were harvested and studied for oxidative response to citrate. Table 3 shows that if there was any effect, the methods employed did not detect it. Citrate dis-

appearance was not investigated in this experiment, although this may have been warranted.

5. The effect of culture age on citrate oxidation

It has been pointed out frequently that the permeability of bacterial cells varies with their cultural age. In general it is maintained that cells in the early log phase of growth are more permeable to various substrates than are physiologically older cells.

To test this possibility with respect to citrate, a thick suspension of resting cells was prepared. Agar slants were inoculated at varying times so that they could be harvested simultaneously, (culture ages were 4,8,16,24 and 48 hr) and tested under identical conditions. The rates of oxidation in the presence and absence of citrate are shown in Table 4. Although the endogenous metabolic activity apparently is considerably higher in young cells, it is not stimulated by citrate at any cell age.

6. Citrate oxidation in the presence of large masses of cells

In view of the relatively small number of cells

TABLE 3

THE RATE OF OXIDATION OF CITRATE BY B. ABORTUS GROWN IN THE PRESENCE OF CITRATE

Substrate	Molarity	pH	$QO_2(N)$
Citrate	0.0033	6.8	99.5
"	0.0033	5.5	75.0
Endogenous	-	6.8	75.0
"	-	5.5	68.0

TABLE 4

THE EFFECT OF CULTURE AGE ON OXYGEN UPTAKE BY B. ABORTUS IN THE PRESENCE OF 0.0033 M CITRATE AT pH 6.8

Cell Age (Hours)	$QO_2(N)$	
	Citrate	Endogenous
4	94	86
8	93	99
16	83	82
24	62	67
48	55	53

generally used for a respiration experiment (approximately 400 µg of cellular nitrogen), it was thought possible that the amount of oxygen taken up in response to citrate may be so small as to be undetectable during the time period of the run. Also, it was previously reported by Altenburn and Housewright[4] that, in the presence of large masses of cells, there was a marked increase in oxygen uptake in the presence of citrate. Therefore an attempt to repeat their experiments was made, following their experimental conditions exactly, i.e., cells were grown for 48 hours on Albimi Brucella agar, washed once in phosphate buffer, suspended in buffer to contain 2 to 3 mg cellular nitrogen per ml, and then aerated for 90 min to reduce endogenous metabolism. A significant increase in oxygen uptake was obtained, as is shown in Table 5. However, as can be seen graphically in Figure 3, this increase is not very dramatic. If the cells were grown and harvested in the usual manner, but used in similarly large masses, no such increase occurred (Table 5). No clean-cut explanation is apparent, although the

TABLE 5

THE RATE OF CITRATE AND cis-ACONITATE OXIDATION BY LARGE MASSES OF B. ABORTUS PREPARED BY TWO DIFFERENT METHODS

Conditions for Preparing cells	Substrate	Molarity	pH	QO_2 (N)
Altenbern and Housewright Method	Citrate	0.015	5.4	62
	cis-Aconitate	0.015	5.4	85
	Endogenous	-	5.4	36
Usual Method (see Methods)	Citrate	0.0033	5.5	109
	cis-Aconitate	0.0033	5.5	132
	Endogenous	-	5.5	106

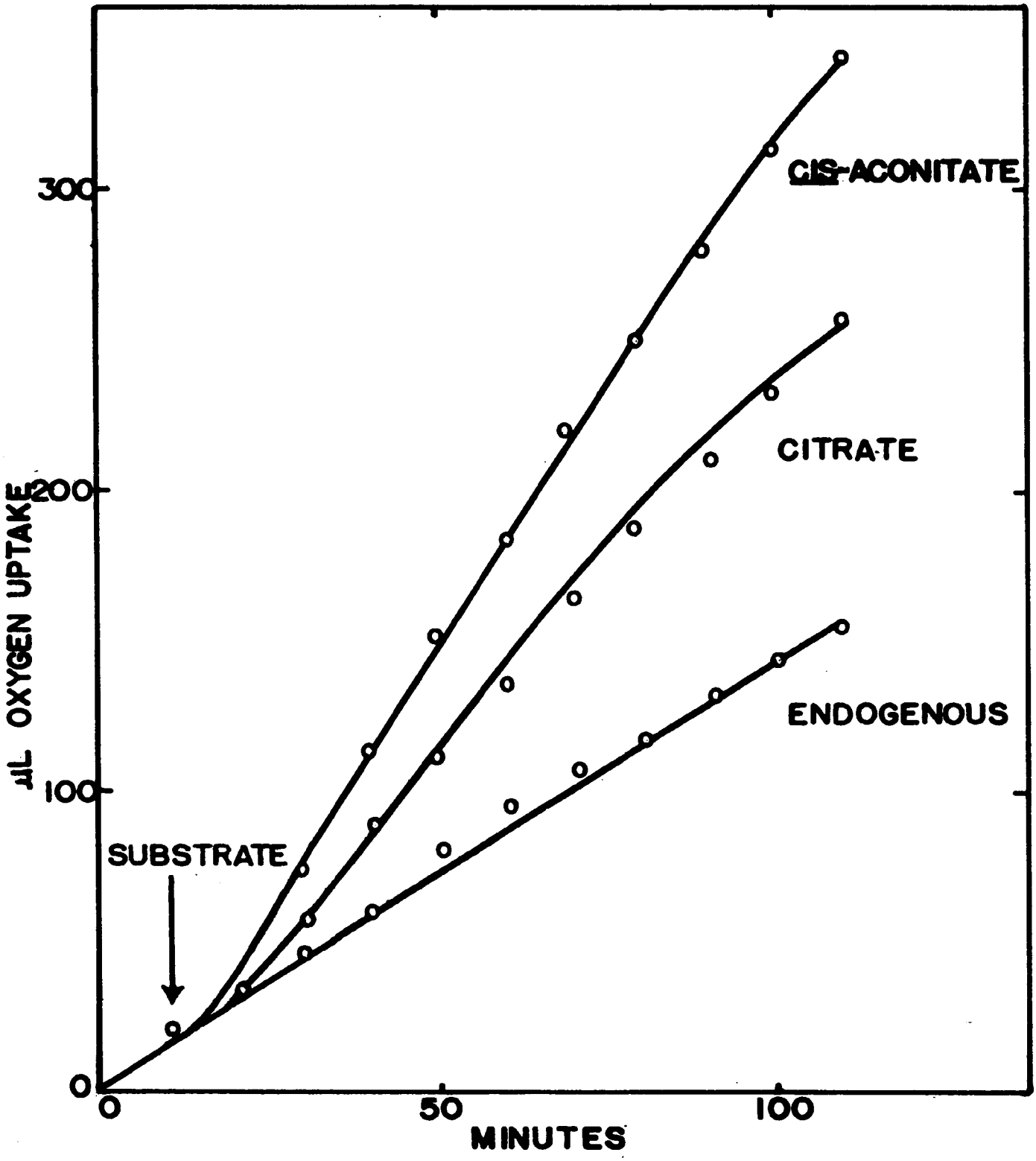


Figure 3. The oxidation of citrate and cis-aconitate (0.015 M) by large masses of B. abortus.

response seems to be tied up with the reduction in endogenous respiration. It may be possible that the cells grown by the Altenburn and Housewright method were partially lysed by the attempt to remove the endogenous respiration and the oxidation obtained was due to enzymes in this cell debris. Experiments on the separate effect of growth on Albimi agar, reduction of the pH to 5.4, use of 48 hr cells, and use of higher citrate concentrations (0.015 M, see Table 5) have shown no increase in oxidation with citrate.

7. The effect of pH of the oxidation of a low concentration of citrate

Erlandson et al.[25,26] have shown that there is a marked response in B. abortus to changes of pH and concentration of succinate, the oxidative optimum occurring at successively lower pH values as the concentration of succinate was decreased. If this were also the case with citrate, one might miss a narrow pH optimum with the two pH levels used. Accordingly, a low concentration of citrate (0.00033 M) was tested at four pH values (pH 4.0, 5.5, 7.0 and 8.0). The data of Table 6 show that

there was no significant rate change from the endogenous at any of the four pH levels. This suggests that pH is not critically involved in the failure of cells to oxidize citrate.

8. The effect of addition of polyvalent metals on the rate of oxidation of citrate

It is well known that many enzymes require polyvalent metals as cofactors for their activity. Also, the chelating ability of citrate has been established and citrate is frequently employed for the removal of certain metals from solutions. If citrate were acting as a chelating agent in the bacterial cell and as such removing a necessary metal cofactor, the non-oxidation of citrate might be explained. To test this idea, a series of experiments was run with equimolar quantities (0.0033 M) of metals and citrate. As is shown in Table 7, none of the metals tried (Co^{++}, Fe^{++}, Mg^{++}, Mn^{++}, Ca^{++}) had the effect of increasing the oxidation of citrate, either in the presence or absence of glucose. The data would seem to indicate that it is not because of metal starvation that citrate it not acted on, but do not indicate whether or

TABLE 6

THE EFFECT OF pH ON THE OXIDATION OF 0.00033 M CITRATE BY B. ABORTUS

pH	$QO_2(N)$	
	Citrate	Endogenous
4.0	45	48
5.5	73	77
7.0	86	98
8.0	98	109

TABLE 7

THE EFFECT OF THE ADDITION OF POLYVALENT METALS ON THE OXIDATION OF CITRATE BY B. ABORTUS

Addition	pH	$QO_2(N)$	
		With Citrate	Without Citrate
Co^{++}, Fe^{++}, Mg^{++}, Mn^{++}, $PO_4^{\equiv}$	6.8	108	105
$PO_4^{\equiv}$	6.8	85	85
Co^{++}, Fe^{++}, Mg^{++}, Mn^{++}, $PO_4^{\equiv}$, glucose	6.8	425	445
Mg^{++}, $PO_4^{\equiv}$	6.8	98	96
Co^{++}, Fe^{++}, Mg^{++}, Mn^{++}, Ca^{++}, $PO_4^{\equiv}$	5.5	69	54
Co^{++}, Fe^{++}, Mg^{++}, Mn^{++}, Ca^{++}, $PO_4^{\equiv}$, glucose	5.5	290	
Glucose	5.5	416	448

not the presence of metals may inhibit the oxidation of citrate.

9. The effect of removal of metals by Versene or cation exchange resin on the oxidation of citrate

Since citric acid chelates various metals, it was considered that perhaps these metals might be bound to the citric acid molecule, making it unable to penetrate the cell wall, to be oxidized by the metabolic system of the cell, or both. If these hypothetical ions were removed by a chelating agent with stronger metal affinity than citrate, citrate may then be oxidized.

A suspension of cells was made in two concentrations (0.01, and 0.001 M) of Versene (ethylene diamine tetra acetic acid) at pH 6.8. After standing for about one hour, the cells were removed by centrifugation and resuspended in fresh Versene, in which condition they remained for 12 hours. Oxygen uptake was studied with these cells in the presence of citrate. The results of this experiment are presented in Table 8. The lack of effect of Versene suggested either that metals were not involved

TABLE 8

THE EFFECT OF THE REMOVAL OF METALS BY VERSENE AND CATION EXCHANGE RESIN ON THE OXIDATION OF 0.0033 M CITRATE BY *B. ABORTUS*

Treatment	Molarity	pH	$QO_2(N)$	
			Citrate	Endogenous
Versene	0.01	6.8	67	68
"	0.001	6.8	67	67
Untreated control	-	6.8	61	66
Nalcite HCR (K cycle)	-	6.8	171	167

in the phenomenon of inhibited oxidation or that the deionization was not complete enough. To test this latter possibility, the entire system was deionized by means of a cation exchange resin.

Nalcite HCR (sodium cycle), a sulfonic acid ion exchange resin (Dowex 50), was converted to the potassium form and washed free of excess hydroxyl ions with deionized water. Cells were suspended in 0.29 M sucrose solution, an equal volume of resin added, and the mixture allowed to stand overnight. The cells were then decanted and used with potassium phosphate buffer which had also passed through this resin. Care was taken to wash all glassware in deionized water. As shown in Table 8, there was no change in the rate of oxygen uptake by cells in the presence of citrate under these conditions. It is concluded that if ions are involved in the inability of cells to oxidize citrate, they are involved at extremely low levels.

10. The effect of citric acid derivatives on oxygen uptake

A series of derivatives of citric acid was investi-

gated for a possible lead to the problem. Trans-aconitate, triethyl citrate, itaconitate, citraconitate and mesaconitate all failed to cause any appreciable change in the rate of oxygen uptake over the endogenous respiration. A more extensive survey of citrate derivatives will be reported in the section on the respiration of E. coli.

11. Non-specific treatments

A number of cell treatments have been reported to be effective in breaking the "permeability barrier" of a variety of organisms. Among those reported were, quick freezing, lypholizing, and drying[1,48,98], addition of cyclopentane dicarboxylic acid[78], addition of insulin[39], or treatment of cells with benzene, chloroform, or toluene[17,28,55]. Each of these treatments was tried with B. abortus but without success in inducing any measurable oxidation of citrate.

12. The oxidation of citrate by cell free preparations

When cells were mechanically disrupted, either in the sonic oscillator or in the Waring blendor, the non-

particulate extract showed a clean-cut ability to oxidize citrate, as is shown in Figure 4. This process requires DPN as a hydrogen acceptor in order to proceed, although it is probable that other acceptors, especially TPN, would do equally well. It is of interest that the estimated $QO_2(N)$ of this extract is about 40. When compared with the rates for large cell masses presented in Table 5 ($QO_2(N)$ of 62 for citrate), this rate would suggest that perhaps the limiting factor in citrate oxidation in B. abortus is the concentration of an enzyme, probably aconitase. This enzyme, if present in only limited quantity could give a very low rate of citrate conversion and thus oxidation, which would not be detected unless the normal high endogenous respiration were suppressed either by aeration or cell disruption.

B. The respiration of Escherichia coli, strain B

1. The oxidative response to various esters of citric acid

It has been reported[7] that certain esters of succinic acid showed a greater tendency to be oxidized

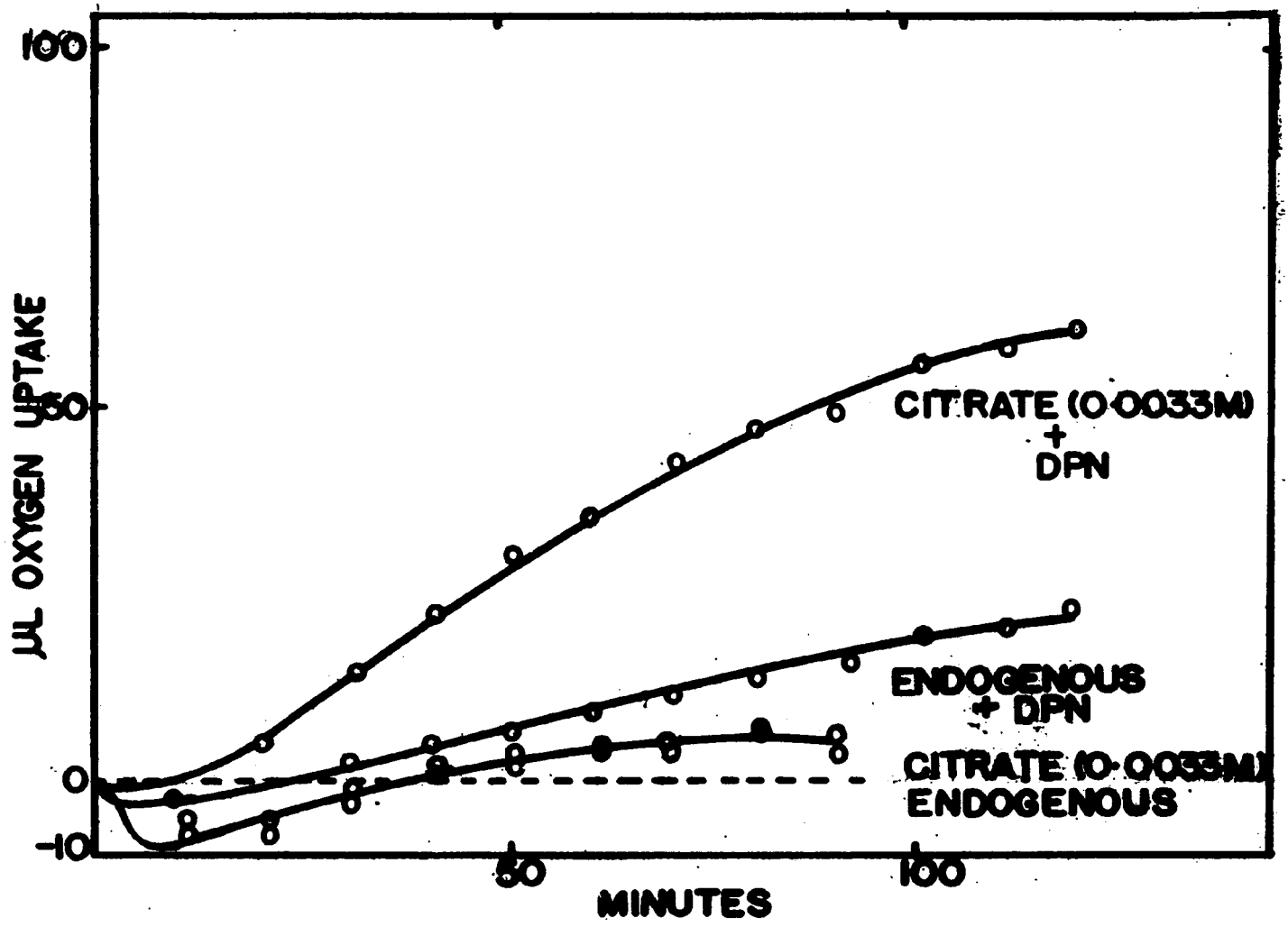


Figure 4. The oxidation of citrate by cell free extracts of B. abortus and its dependence upon diphosphopyridine nucleotide (DPN, 0.5 mg/flask; Nutritional Biochemicals, "Chromatographically pure").

by Penicillium chrysogenum than succinic acid itself. It was suggested that these esters were more permeable because of their lesser polarity. It seemed possible that citrate, because of its high polarity, similarly might be unable to penetrate the cell membranes. A series of mono-, di-, and tri-substituted citrate esters was therefore obtained and tested with E. coli. Each ester was tested at pH 6.8 and 5.5 at a concentration of 0.0033 M, except for the more highly substituted ones which were not very soluble. The QO_2 values of these compounds are given in Table 9. There was no significant increase in the rate of oxygen uptake with any of the esters used, although acetyl-triethyl-citrate caused a considerable depression of this rate. There is no a priori reason why the citrate oxidizing enzymes should oxidize these esters even if they did succeed in reaching the enzyme sites. Since direct determination of uptake with these esters was not made this experiment does not necessarily rule out the possibility that citrate may be excluded because of its polarity. Contrary to our

TABLE 9

OXYGEN UPTAKE IN THE PRESENCE OF A SERIES OF CITRIC ACID ESTERS BY E. COLI

Group	Ester	Mol wt.	Molarity	QO_2 pH 6.8	QO_2 pH 5.5
	Monomethyl citrate	206.13	0.0033	9.63	7.88
	Monoethyl citrate	220.14	0.0033	10.00	8.10
	Monobutyl citrate	248.16	0.0033	9.29	6.53
I	Dimethyl citrate	220.14	0.0033	10.00	7.61
	Diethyl citrate	248.16	0.0033	9.57	6.96
	Trimethyl citrate	234.15	0.0033	9.57	7.77
	Triethyl citrate	276.18	0.0033	8.05	6.64
	Endogenous for Gr.I	-	-	9.73	7.43
	Tributyl citrate	356.24	saturated	9.86	8.15
	Tribenzyl citrate	413.13	saturated	7.38	6.14
	Acetyl triethyl citrate	318.14	saturated	5.62	3.48
II	Acetyl tributyl citrate	402.20	saturated	8.19	6.74
	Acetyl trihexyl citrate	486.26	saturated	8.81	8.96
	Acetyl tri-2-ethylhexyl citrate	570.32	saturated	8.58	7.01
	Citric acid	192.12	0.0033	10.00	8.59
	Endogenous for Gr.II	-	-	8.00	7.55

findings, Gunsalus (personal communication to Dr. P. Gerhardt) has reported that E. coli is able to oxidize tributyl citrate.

2. Induction of citrate oxidase activity by heating

Recent work[29,40,41,42,43,88] has indicated that some enzymes may occur in normal cells in a state of reduced activity. Kaplan[29,40,41,42,43] suggested that yeast catalase is much more heat stable in this state than in the fully active state. He has, furthermore, been able to re-stabilize this enzyme by adsorbing it in a lipid emulsion, whereby its activity is again reduced. Swartz et al[88] on the other hand have suggested that the heat stability of some "masked" enzymes is due to the presence of an inhibitor group which is thermo-labile and which can be removed by heating.

For many years it has been known that some enzymes are more heat stable than others, and in bacterial spores certain enzymes require heating before they are active[13]. In view of this information it was thought that perhaps

the citrate oxidase is present in the cell in a "masked" state, either because of an inhibitor or because of chemical configuration; this masking might be removable by heating, provided that the heat did not destroy the enzyme. Another possibility considered was that the citrate oxidase is present, but is either inhibited or obscured by the endogenous respiration; by heating, these endogenous enzymes may be inactivated at a greater rate than the citric oxidase system so that the latter can be detected.

Cells were heated at 100^{o} for short periods varying from 5 to 20 seconds, and oxygen uptake then observed in the presence and absence of citrate. As shown in Figure 5, at 12 or 14 seconds of heating the endogenous respiration was at a minimum, whereas in the presence of citrate, cells took up oxygen at a much greater rate. At 11 or 15 seconds much less effect was observed. When the effect of heating on QO_2 is plotted against the length of the heat treatment (Figure 7) it is observed that the endogenous inactivation rate is no steeper, but

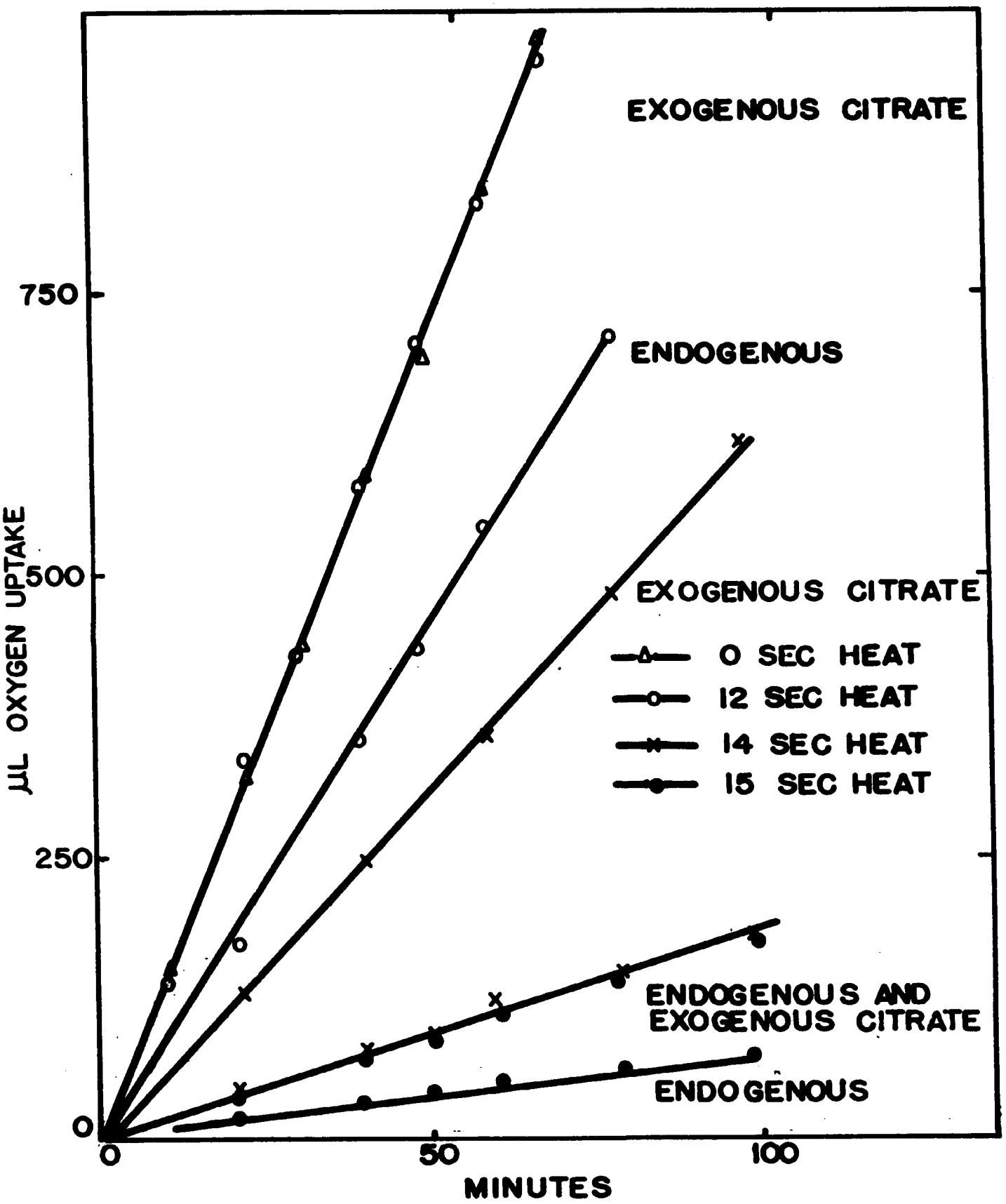


Figure 5. The effect, on the respiration of E. coli in the presence and absence of citrate, of heating 12, 14 or 15 seconds at 100°C.

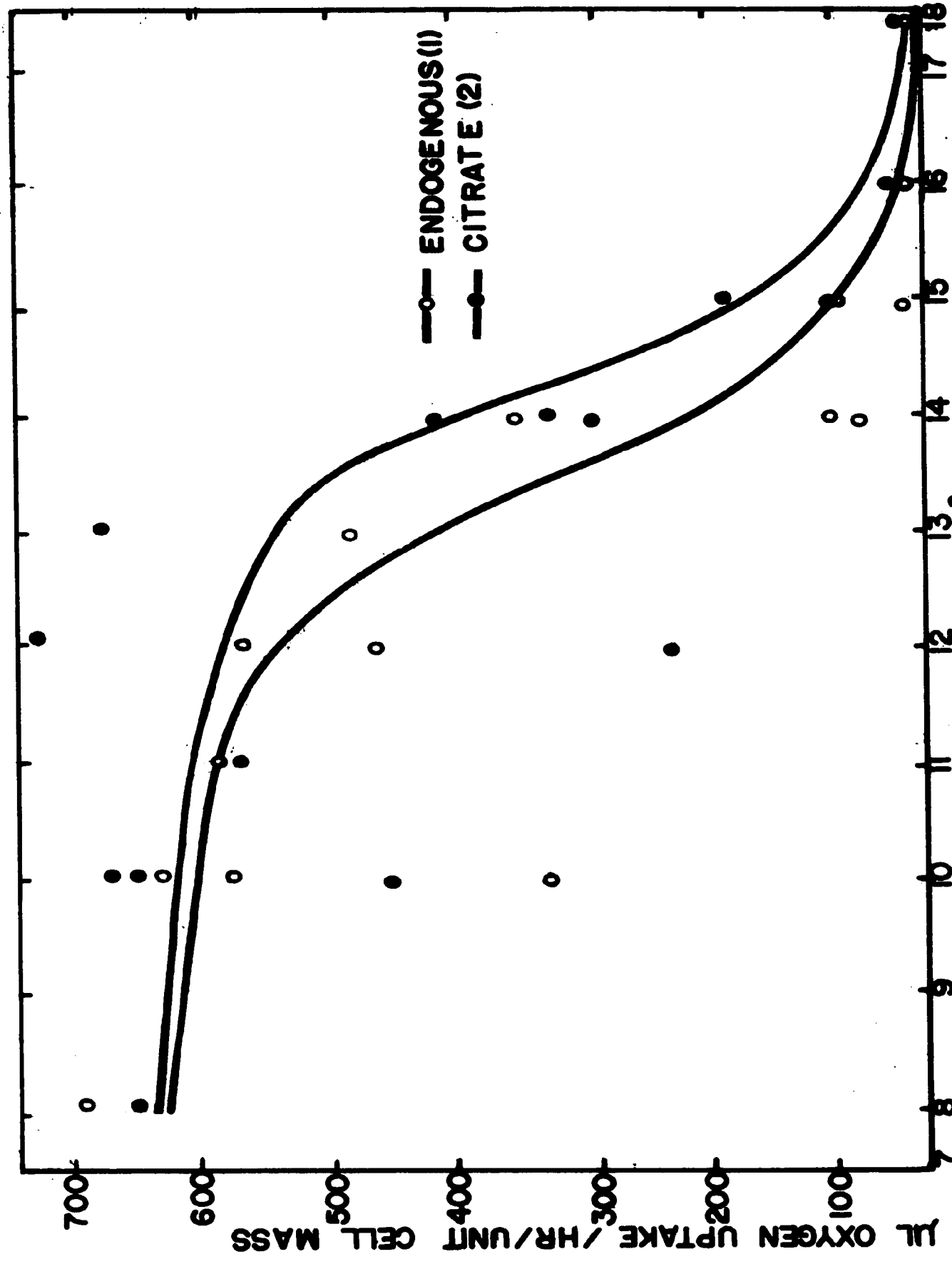


Figure 6. The effect of heating on the rate of citrate oxidation and endogenous respiration of E.coli

begins sooner than the citrate oxidation inactivation rate. It is to be noted in Figure 6 that the spread of points between the critical times is very great and the solid lines are only meant to suggest a possible kinetic interpertation. This results partially from the fact that the effect is only observed over a two second time range, making replication very difficult since rate of heating up and cooling off cannot be rigidly controlled by the methods used. Other factors, which were not controlled, such as light intensity and cell age could be important here as well. Therefore this kinetic interpretation (which is actually the best fit of the average values) must be considered as only tentative. Moreover, repetition of this experiment is difficult because it is necessary to determine the optimum temperature for each batch and concentration of cells.

3. The effect of various cell treatments on citrate oxidation

Lara and Stokes[48] reported that E. coli was able to oxidize citrate if the cells had been previously dried

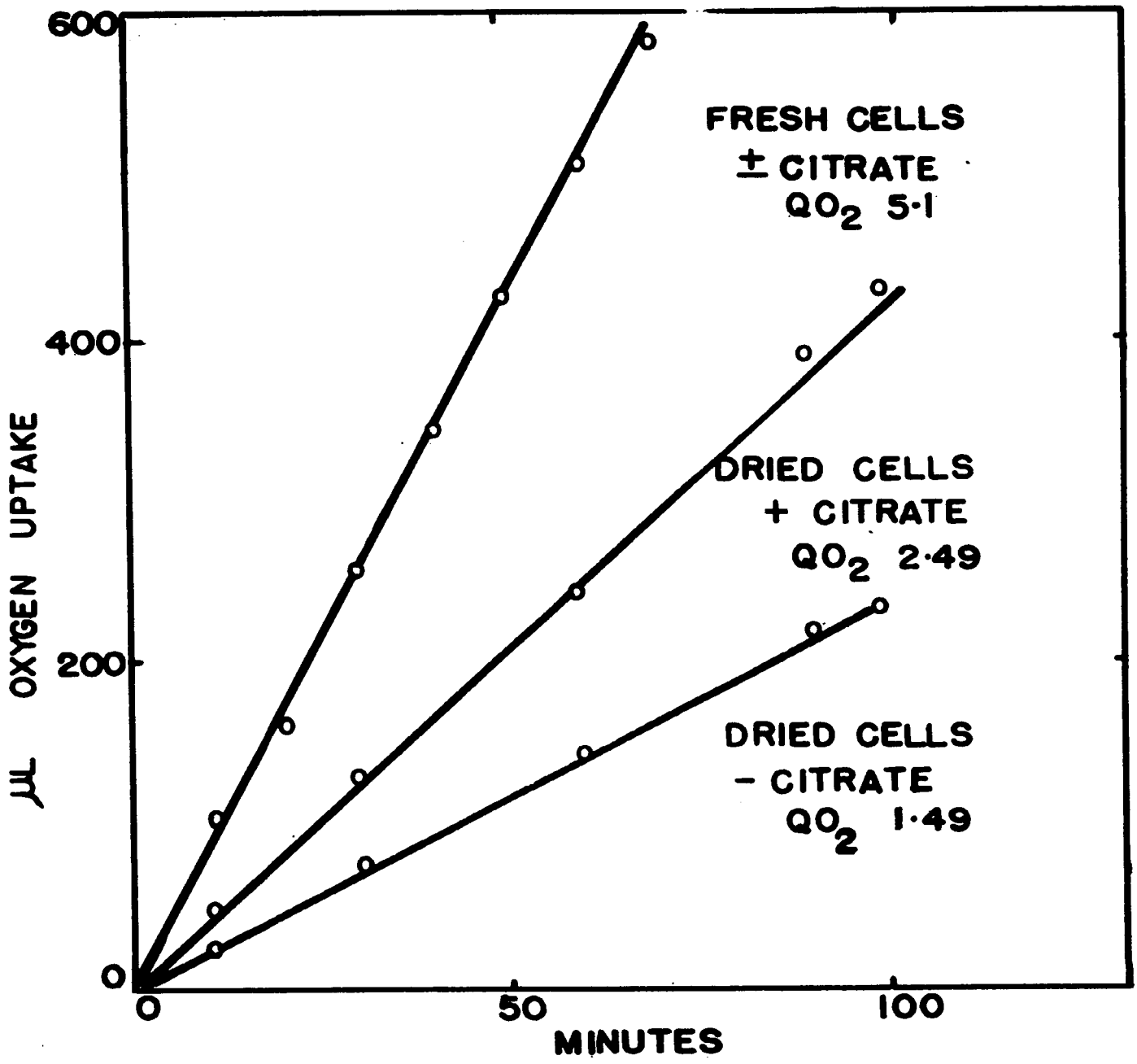


Figure 7. The oxidation of 0.03 M citrate by dried cells of E. coli at pH 6.8, 34°.

over sulfuric acid or lypholized. This observation was confirmed as is shown in Figure 7. Other treatments, however, such as freezing, butanol or toluene extraction, lowering or raising the pH, or addition of Fe^{+++} ions did not cause any measurable change in the rate of respiration either in the presence or absence of citrate. Cell extracts, on the other hand, prepared by means of sonic oscillation or by treatment with lysozyme and versene did show an oxidative response to the addition of citrate.

II. The uptake of citrate by B. abortus and E. coli

A. Measurement of intercellular space

1. The rate of packing of E. coli in a high speed centrifuge

As Conway and Downey[15] have pointed out, the amount of space between cells after packing is dependent on the degree of the packing. The longer that cells are packed in a centrifuge at a given force, the smaller the intercellular space will be. It is necessary, therefore, to know the rate of packing in order for a reproducible intercellular space value to be obtained.

Cells were suspended in 0.067 M phosphate buffer at pH 6.8. Equal volumes were pipetted into tared centrifuge tubes and made up to about 30 ml in volume. Each tube contained approximately 5 gm of cells (wet weight). These tubes were then centrifuged at 20,000 G for the required length of time in a Servall SS-1 centrifuge. The liquid was then replaced and the cells were recentrifuged without resuspension, for the desired time. This time was considered additive as in shown in Figure 8. There was an initially rapid decrease of pack weight followed by a slower rate of decrease, which never levelled off. This suggested that on prolonged centrifugation, extensive cell rupture occured. Such a possibility is supported by the continual occurrence of trichloroacetic acid-precipitable materials in the supernatant. It is therefore important to keep the time of centrifugation to a minimum. For later experiments, 20 min was chosen as the shortest easily measurable time at which the curve is sufficiently flat to be reproducible.

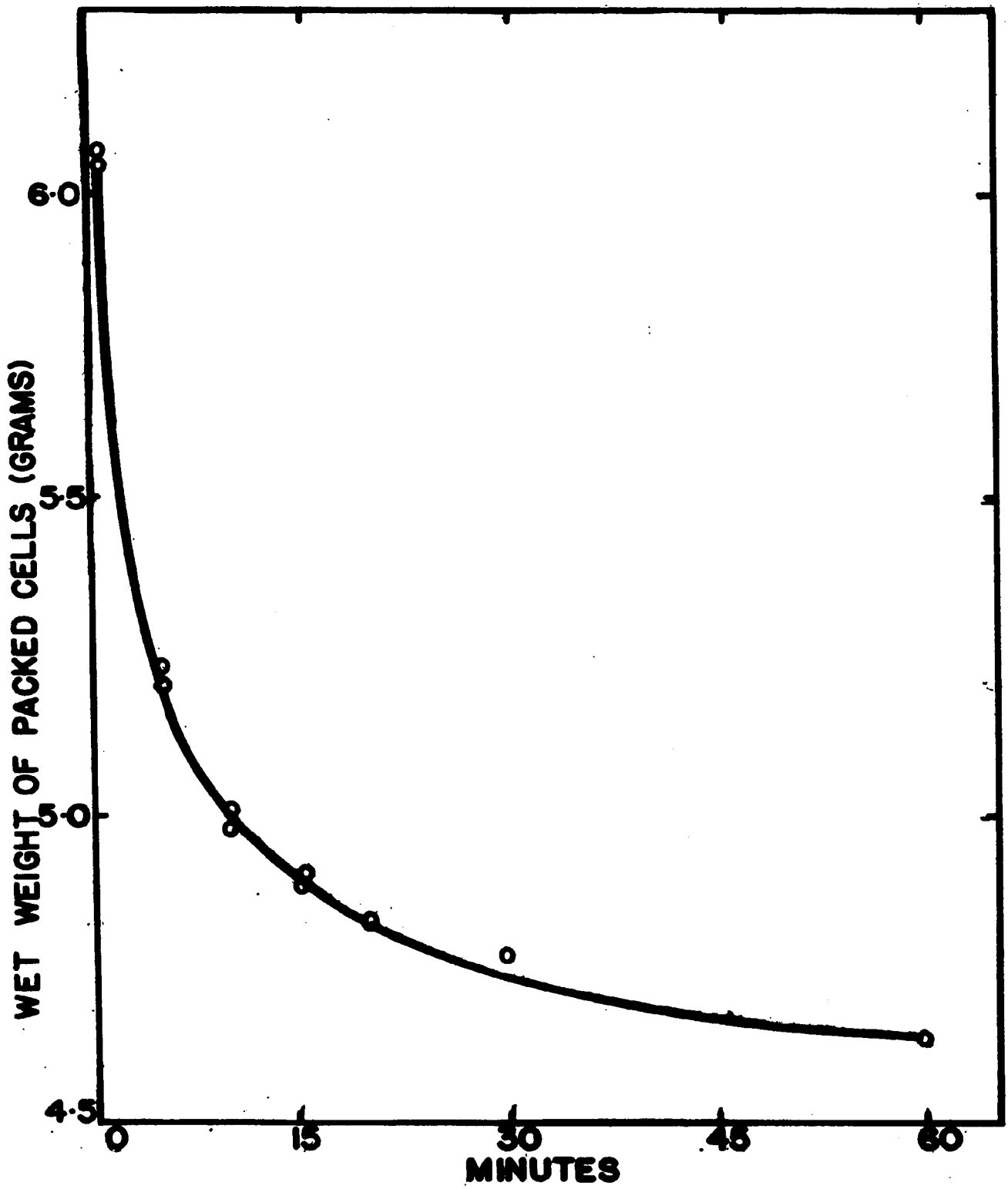


Figure 8. The rate of packing of E. coli at 20,000 G in the Servall SS-1 centrifuge.

2. The intercellular space of E. coli and B. abortus

In order for determinations of the intercellular space to be useful they must be reproducible, since it is not practicable to measure it in every experiment. Cells were grown in a carboy as described in Methods, washed twice, packed for 20 min at 20,000 G in the centrifuge, and re-suspended in a solution of inulin. A series of determinations using separate preparations of E. coli was made and the inulin space determined. Table 10 shows the results of these experiments. The mean intercellular space is 6.7%. The probability of a one percent deviation from the mean in a single tube estimation is less than 5%. This makes it unnecessary to run more than a few intercellular space determinations for a given set of conditions.

Intercellular space determinations with B. abortus were necessarily limited in number, due to the hazard of handling dried supernatants from these cells. Large masses of B. abortus cells were grown, harvested, and washed. They were packed at 20,000 G for 30 min, and

TABLE 10

THE INTERCELLULAR (INULIN) SPACE OF *E. COLI*: CENTRIFUGED FOR 20 MIN AT 20,000 G[1]

Pack weight Wp(gm)	Substrate volume, Vs(ml)	Initial substrate concentration, Co(mg/ml)	Final substrate concentration, Cf(Mg/ml)	Inulin space of cells,[2] Sin(%)
7.22	8	129.4	122.2	6.9
7.23	8	129.4	121.5	7.2
7.56	8	129.4	121.6	6.9
7.32	8	129.4	122.3	6.6
7.70	8	129.4	122.5	6.0
7.75	8	129.4	123.2	5.2
7.31	8	129.4	122.3	6.6
7.39	8	129.4	122.5	6.4
7.22	10	200.4	189.0	6.9
7.23	10	200.4	189.7	7.2
7.56	10	200.4	188.9	7.0
7.32	10	200.4	188.1	7.6

Mean($\bar{X}_R$) = 6.7%; Variance(s_R^2) = 0.0035; Standard deviation (s_R) = 0.6

[1]The abbreviations and units given here will be used in subsequent tables and figures.

[2]For simplicity Sin values are expressed as ml/gm x 100 or percent.

then resuspended in inulin for space determinations. The mean space of packed cells was 9% for four determinations (see Table 11).

B. Measurement of intracellular space

1. The citrate space of B. abortus

B. abortus cells grown and prepared as described above were packed for 30 min at 20,000 G. They were resuspended in a small volume of 0.067 M phosphate buffer at pH 6.8, a known concentration of citrate was added, and the mixture incubated at 0° for 10 min. The data presented in Table 11 indicates that citrate enters about 41% of the cell[1]. This is too small a value to conclude that there is free entry of citrate into all parts of the cell but it is large enough to suggest that at least some of the cell internal to the cell wall is penetrated. The data in Table 11b is an extension of 11a and shows that all of the citrate lost is recoverable by

[1]The high degree of variation (s.d. 14.9) was caused by the citrate analysis used. The average value, however, is felt to be reasonably accurate on the basis of similarly averaged values with E. coli, which come within one percent of the value found by more accurate analysis.

TABLE 11

THE CITRATE SPACE OF B. ABORTUS

Pack weight Wp(gm)[1,2]	Substrate volume Vs(ml)[1]	Intercellular space of pack Sin(ml/gm)[1]	Initial substrate conc. Co(mg/ml)[1]	Final substrate conc. Cf(mg/ml)[1]	Citrate space R(ml/gm x 100)[1,3]
12.57	15	0.080	25.6	17.2	54.9
12.57	15	0.080	25.6	18.2	48.6
6.50	15	0.115	25.6	22.0	29.8
6.50	15	0.115	25.6	20.6	50.2
6.50	15	0.115	25.6	22.6	21.5

Mean($\bar{X}_R$) = 41.0; Variance (s_R^2) = 207.9; Standard deviation (s_R) = 14.9

[1]The abbreviations and units given here will be used in subsequent tables and figures.

[2]Two tubes of cells were used. From one the supernatant was analyzed in duplicate, from the other in triplicate. The fluctuation of the Cf values is apparently due to the methods of citrate analysis, therefore the R values may not be valid.

[3]Sample calculation: $Scit = \frac{Vs}{Wp}(\frac{Co}{Cf}-1) = \frac{15}{12.57}(\frac{25.6}{17.2} - 1) = 0.584$

$$R = \frac{Scit - Sin}{1-Sin} = \frac{0.584-0.080}{1-0.080} = \frac{0.504}{0.92} = 0.549$$

$$R = 54.9\%$$

TABLE 11b

CITRATE RECOVERABLE BY WASHING[1]

Initial volume of wash water V(ml)	Volume of wash + vol. of cells V + Wp(gm)	Amount of substrate in pack G(mg)	Expected recovery[2] (mg)	Found (mg)	% Recovery
10	16.5	60	36.8		
10	16.5	75	45.5		
			$\bar{X}$ = 41.1	42.2	98.0

[1] Percent recovery from three washes = 101% of that lost from the supernate

[2] Expected recovery = $\frac{V}{V + W_p} \times G$

washing. Due to difficulties in handling such large quantities of pathogenic organisms, further experimentation was not done with B. abortus.

2. The citrate space of E. coli

(a) The citrate space at 0° in 0.067 M phosphate buffer at pH 6.4[1]

E. coli cells for this experiment were grown by the carboy method, washed three times with buffered saline (pH 6.8)[1] and then packed and treated as outlined in Methods. Seven tubes of cells were tested, as shown in Table 12. A mean citrate space of 22.8% with a standard deviation of 1.1% was obtained. This was considered to be a close check on the accuracy of the method under the conditions used. As will be shown later, this space value is variable according to the conditions used. Under the above mentioned conditions the space is only about one-half that found for B. abortus, and is by no means clean-cut evidence that citrate is penetrating the cell to any extent.

[1]The apparent discrepancy between the pH of the experiment in the heading and the pH of the buffer used was due to the cells: even after several washings with pH 6.8 buffer, direct pH determinations on the cell suspensions gave the lower (pH 6.4) value.

TABLE 12

THE VARIATION IN DETERMINATION OF CITRATE SPACE IN *E. COLI*

W_p (gm)	V_s (ml)	C_o (mg/ml)	C_f (mg/ml)	R
8.01	5	2.000	1.380	22.8
8.46	5	2.000	1.364	22.1
8.06	5	2.000	1.380	22.9
8.05	5	2.000	1.408	23.0
8.32	5	2.000	1.404	21.8
8.02	5	2.000	1.380	22.6
8.07	5	2.000	1.312	26.3

$\bar{X}_R = 22.8$; $s_R^2 = 0.0244$; $s_R = 1.1$

(b) A comparison of the citrate space with the sulfate and pyruvate spaces

Roberts et al.[70] have stated that space values in E. coli are constant for a large number of substrates. They suggested, on the basis of this evidence, that the cell is unable to distinguish between small molecules. The low values obtained with citrate in the preceeding experiment suggested that perhaps an exception was present in the case of the citrate molecule, although Gerhardt (unpublished results) was unable to repeat the high (75%) space values they obtained even though following their methods exactly. Accordingly an experiment to compare citrate with one of the molecules (sulfate) tested by Roberts et al. was undertaken. Isotopically labelled potassium sulfate ($K_2 S^{35} O_4$) was employed in conjunction with citrate. Each substrate was present at the same time so that any variations between the two determinations, if done separately would be eliminated. Pyruvate was used in a separate experiment as a readily metabolized compound which

should freely enter the cell. A comparison of these three compounds is shown in Table 13. Sulfate and citrate appear to have entered identical volumes of the cell when they both were present. However if no citrate was present the sulfate space dropped slightly but significantly. Other experiments (Gerhardt, unpublished results) show that the sulfate space in the presence of pyruvate had a mean value of 32.6%. It may likewise be pointed out that the results in Table 12 were obtained in the absence of sulfate. The higher values obtained in the present experiment may thus represent a stimulation produced by the sulfate. This will be considered in a later experiment (section g).

The pyruvate space shown in Table 13 is not too significant, other than as a demonstration of the effect of metabolism on space since even at the lowest temperature employed (0°), enough metabolism could have occurred during centrifugation when the temperature probably rose, to account for this high value.

TABLE 13

A COMPARISON OF THE SPACE AVAILABLE TO SULFATE, CITRATE, AND PYRUVATE IN E. COLI

Substrate	Wp (gm)	Vs (ml)	Co (mg/ml)	Cf (mg/ml)	R
Citrate[1]	9.8	15	0.667	0.530	35.0
"	10.4	15	0.667	0.528	33.4
"	9.8	15	0.667	0.524	37.9
Pyruvate (0^{o})	9.8	15	0.667	0.460	65.3
"	10.4	15	0.667	0.380	106.0
Pyruvate (37^{o})	9.8	15	0.667	0.000	-
"	8.9	15	0.667	0.000	-
Sulfate[2]	9.8	15	131.5[4]	104.2	35.6
"	10.4	15	131.5[4]	103.1	35.4
"	9.8	15	131.5[4]	104.2	35.9
Sulfate[3]	9.7	15	131.5[4]	106.8	31.3

[1,2] Both substrates citrate and sulfate run concurrently

[3] Sulfate determination in the absence of citrate

[4] Concentration given in counts per min x 10^{-3} using isotopically labelled potassium sulfate ($K_2S^{35}O_4$).

(c) The effect of energy sources

The ability of E. coli to break down citrate in the presence of an energy source such as glucose[18,50,52,91,95] suggests that glucose may act in allowing citrate to cross an impermeable membrane. An experiment undertaken to study the effect of an energy source, however, showed no significant difference in citrate space in the presence or absence of glucose at any of the temperatures used. The data are given in Table 14. In view of the high concentration of cells it is possible that there may not have been enough glucose present to produce an effect, but this seems unlikely. Equimolar concentrations of isocitrate were added to determine whether there possibly was competition for certain sites in the cell. The closeness of the space values in its presence and absence, however, suggest that there is no competition.

(d) The effect of temperature

If the citrate space measured at 0^{o} were dependent on a metabolic process, it ought to be greatly increased by an increase in temperature. Moreover it ought either

TABLE 14

THE EFFECT OF AN ENERGY SOURCE ON THE CITRATE SPACE IN *E. COLI*

Energy source	Molarity	Temperature $0^{o}C$	R^{1}
Glucose	0.05	0	22.0(21.0)
Glucose	0.005	37	19.0(21.0)
Glucose	0.011	0	18.0(20.0)
Glucose	0.011	34	25.0(22.6)
Glucose	0.011	50	31.0(27.2)
Isocitrate	0.01	34	18.0(22.6)

[1] Space values in parentheses are control values in the absence of an energy source

to decrease markedly at high temperatures (60^{o}) at which most enzymes are inactivated, or to increase markedly due to the death of the cells and subsequent disruption of the barriers which restrict permeation. On the other hand if the space being measured is one that depends on diffusion, no marked change should occur, although one would expect an upward trend as temperature increased. Space due to adsorption would tend to decrease as temperature increased. As shown by the data in Table 15 and the solid line in Figure 9, the former effect is obtained. Although this experiment does not necessarily exclude the possibility of an adsorption phenomenon it is more in agreement with the theory that the space is due to free diffusion. In two other experiments, there was an abrupt increase in space at temperatures above 40^{0}; the maximum space observed was about 65% at 50^{0}, as is shown by the broken line in Figure 9. The reason for this abrupt increase is not understood in the light of present information on the nature of the space in cells. Attempts to repeat these observations under the

TABLE 15

THE EFFECT OF TEMPERATURE ON THE CITRATE SPACE OF *E. COLI*

Temperature ^{o}C	Wp	Vs	Co (mg/ml)	Cf (mg/ml)	R
60	7.81	5	2.000	1.156	43.0
60	8.33	5	2.000	1.166	38.7
50	8.36	5	2.000	1.234	32.2
50	8.18	5	2.000	1.236	33.1
45	8.50	5	2.000	1.236	31.5
45	8.04	5	2.000	1.236	35.0
0	8.02	5	2.000	1.380	22.6
0	8.07	5	2.000	1.312	26.6

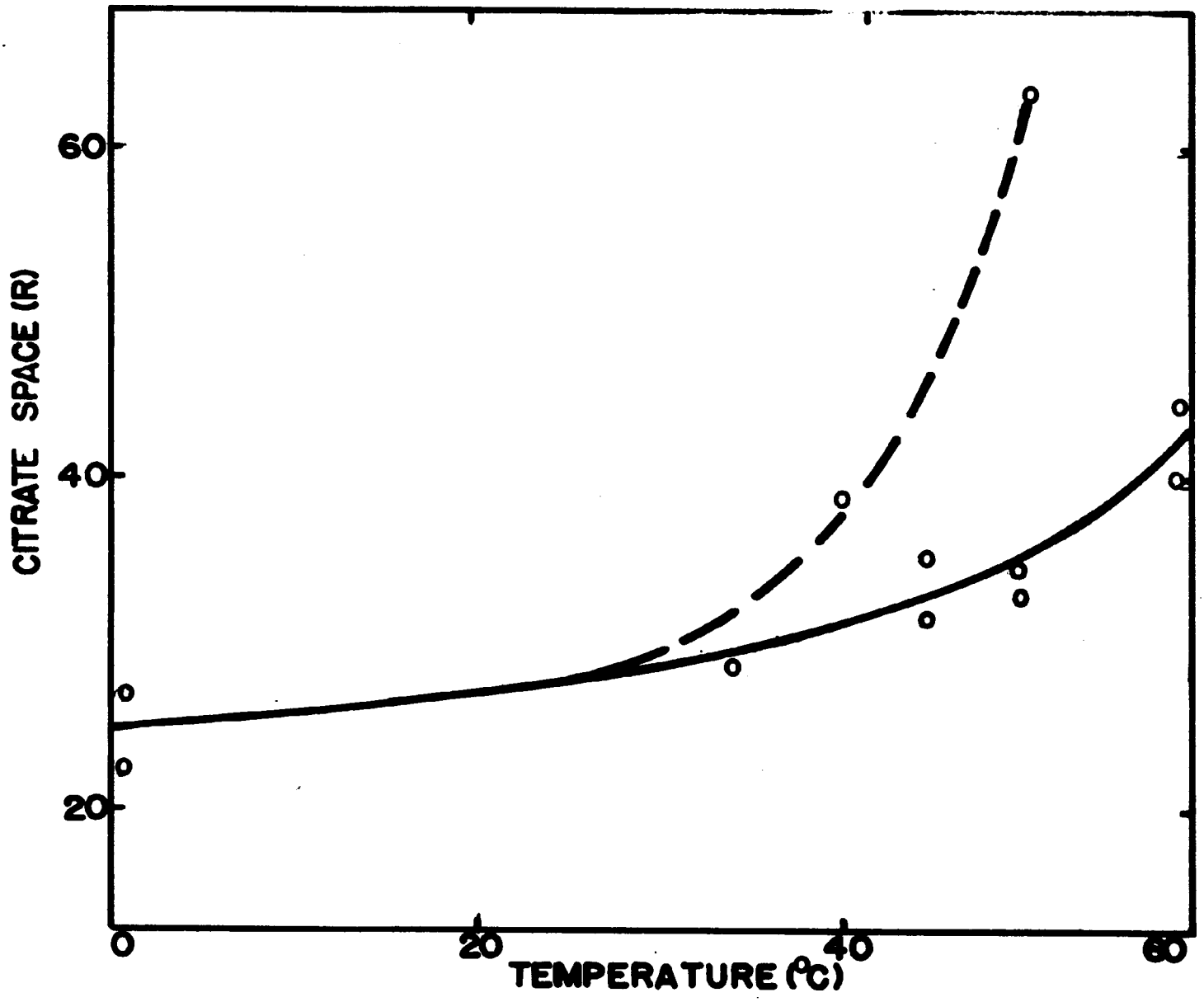


Figure 9. The effect of an increase of temperature on the citrate space of *E. coli* at pH 6.6.

same or varied conditions were unsuccessful. The experiments on "heat activation" (Section B-2) may offer a possible explanation for this marked increase in space in that the cells may now respond to citrate oxidatively.

(e) The effect of pH

It has been shown by a number of workers[26,80,81,84] that the pH exerts a marked effect on the rate of metabolism of various substrates by intact cells and it has been suggested[26,27,84] that in some cases this pH response is due to differences in cellular permeability to dissociated or undissociated molecules. Although changes in pH did not have any effect on the oxidative response of E. coli to citrate, it was of interest to determine whether there was any effect on the amount of citrate penetration into the cells. Thus, the citrate space was determined on cells at a series of pH's from 3.0 to 9.0. The results of this experiment are reported in Figure 10. Cells were grown in the usual manner in a carboy, and distributed in centrifuge tubes. Here they were washed twice with phosphate buffer at the desired pH (pyrophos-

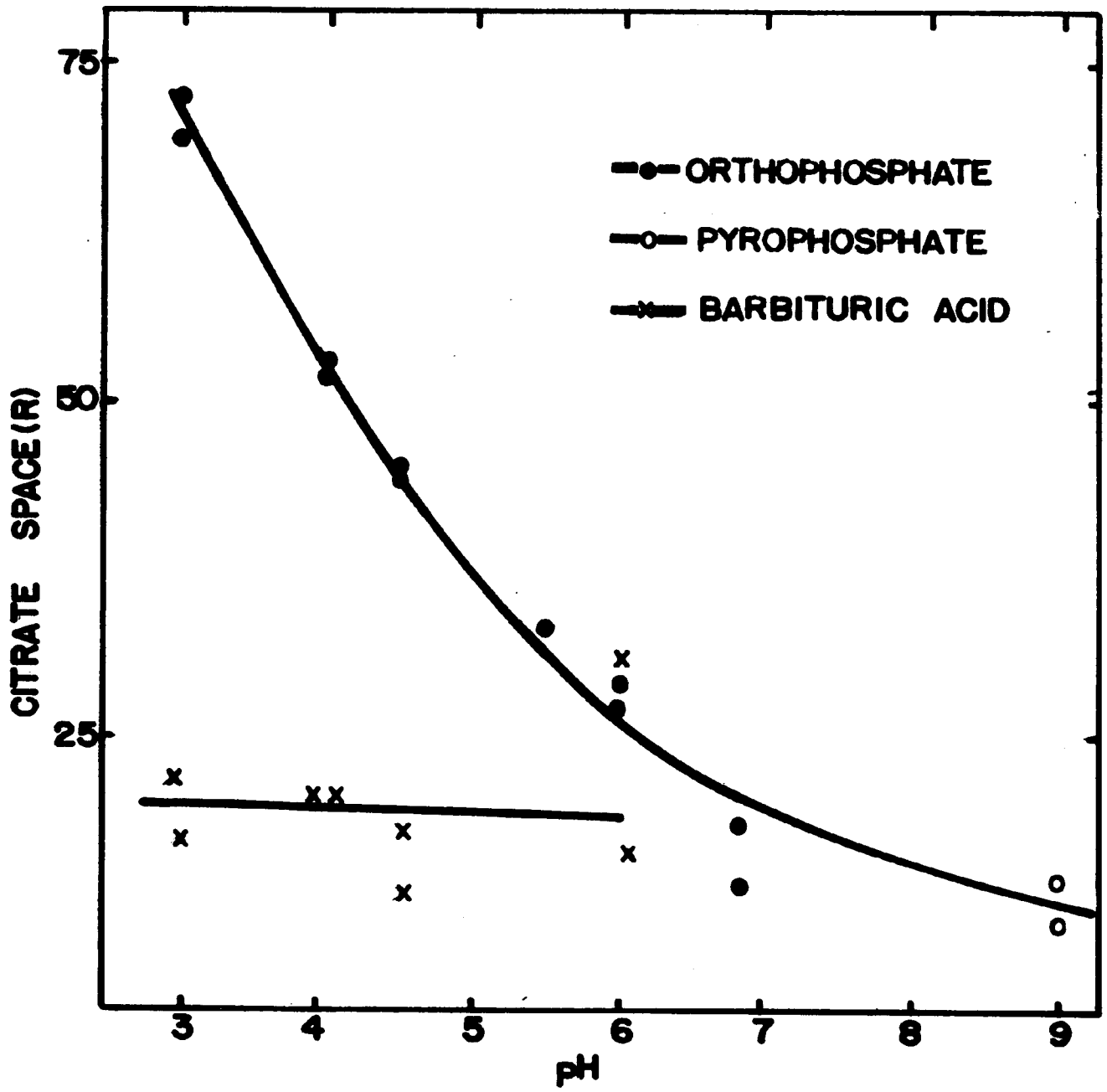


Figure 10. The effect of pH on the citrate space in E. coli using different buffers at a molarity of 0.067. Citrate concentration 0.2 mg/ml.

phate was used for pH 9.0) and then the mixture was adjusted with phosphoric acid or potassium hydroxide to the required pH. These cells were now packed in the centrifuge and the space was determined as before using citrate solutions at the desired pH's. As is shown in Figure 10, the effect of low pH on citrate uptake is marked, the citrate space rising abruptly from about 22% at pH 6.0 to about 70% at pH 3.0. This increase may be due to the greater ease of penetration of the undissociated molecule or to the effect of pH on the cell itself. An effect on the cells, if present is not permanent since on return to higher pH values there is a subsequent decrease in space. The effect of pH seems to be dependent on the type of buffer used, for if barbituric acid buffer (0.067 M) is substituted for the phosphate buffer, no change in space was observed over a pH range of from 3.0 to 6.0. This would suggest that the cells respond to pH in the presence of phosphate, but not in the presence of other ions. This possible ion involvement will be considered in a later section. Alternatively, barbiturate

may have been lethal to the cells but, if so, higher space values should result rather than lower. Cell viability was not determined.

(f) The effect of citrate concentration

If the space values observed with citrate are due only to adsorption by the cell surfaces then, as one increases the concentration of citrate, the space value should fall as the adsorptive sites become saturated, since the ratio of Co/Cf would decrease. This decrease in space value would be caused by the fact that as saturation of adsorption sites for a given number of cells occurred with increasing Co, Co-Cf would become equal to a constant amount and consequently Co/Cf would decrease and approach 1. On the other hand if the externally added substrate were equilibrating with the internal cell water, the space should remain independent of the increasing concentration. A possible divergency from this rationale could be caused by an outflow of water from the cell as a result of osmotic pressure. This would dilute the substrate in the same manner as would its entry into the

cell. The error could be correct by analyzing the cell pack for citrate content, except that, at the concentration of citrate at which this effect would begin to be important, cells will not form a firm pack on centrifugation.

Table 16 shows the effect of concentration on citrate space. These data are presented graphically in Figure 11. At low levels of citrate, space tends to decrease but as the concentration is increased the space increases until it reaches a plateau and remains constant. The signifigance of these results is not clear but may represent two different occurrences: first, an adsorption phenomenon and second, diffusion into the cell, the latter occurring only after the saturation of the adsorption sites on the cell.

If the data given in Table 16 are replotted (Figure 12) it is found that there is a linear relationship between the concentration of citrate in the test solution and the amount lost to the cell pack. This suggests that adsorption (which should give a saturation curve) is not

TABLE 16

THE EFFECT OF CITRATE CONCENTRATION OF CITRATE SPACE IN *E. COLI*

Wp	Vs	Co(mg/ml)	Cf(mg/ml)	R
5.5	4	1.0	0.580	49.5
5.6	4	1.0	0.575	49.0
5.9	4	2.0	1.150	46.8
5.9	4	2.0	1.160	45.7
5.7	4	10.0	6.800	28.0
5.2	4	10.0	7.000	28.2
6.1	4	20.0	12.600	23.8
6.1	4	20.0	13.300	27.4
5.9	4	50.0	28.800	45.7
5.7	4	50.0	28.250	50.5
5.8	6	66.66	43.200	52.2
5.7	6	66.66	45.760	37.6
5.9	6	83.33	56.000	46.3
5.8	6	83.33	58.500	63.5

Cells suspended on 0.05 M $CaCl_2$, pH approx. 6.0 at 0 °C.

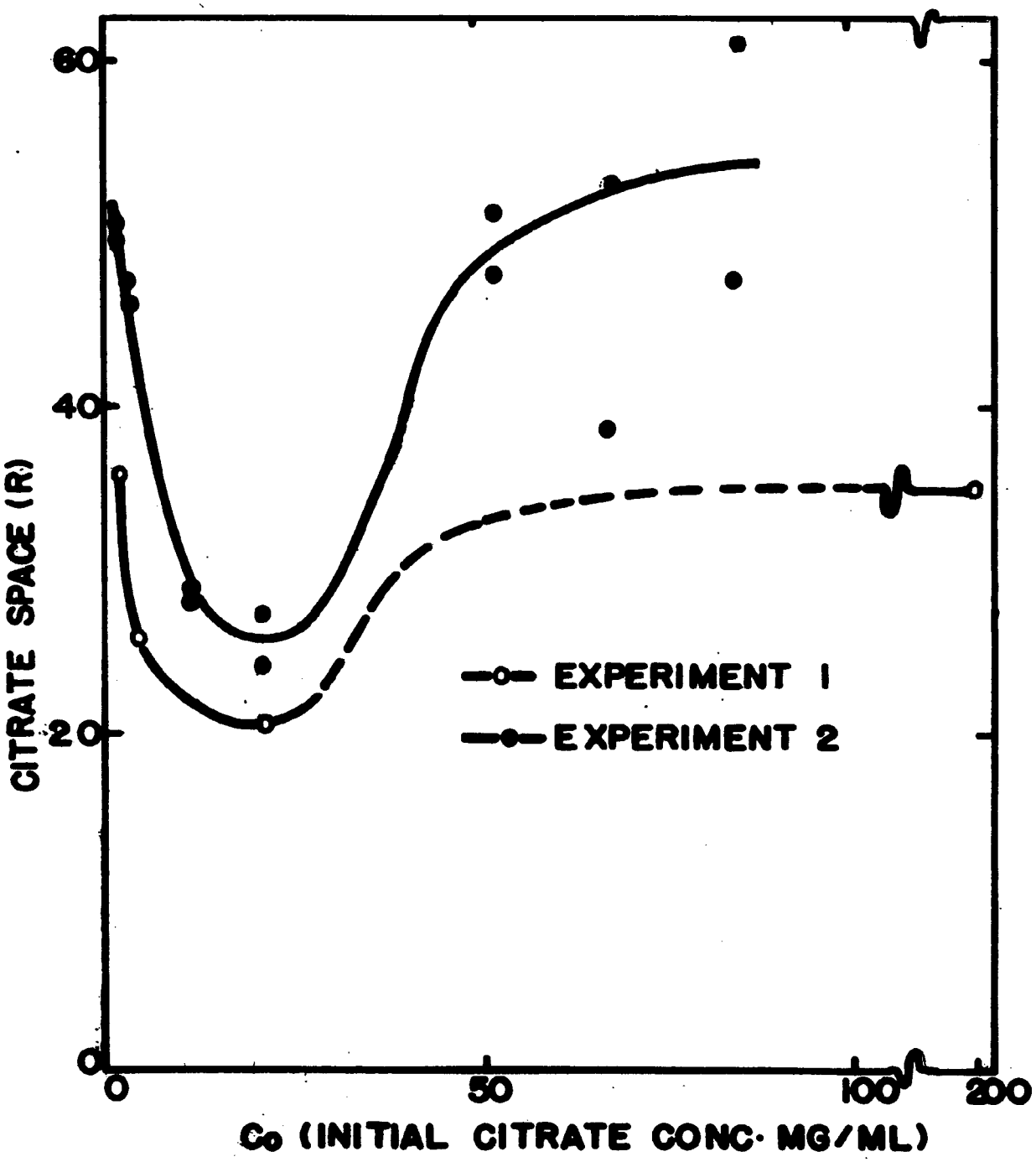


Figure 11. The effect of citrate concentration on the citrate space of *E. coli*. pH of Expt 1, 6.8; Expt. 2, 6.0. Expt. 1 carried out in 0.067 M phosphate buffer + 0.9% NaCl. Expt. 2 carried out in 0.05 M $CaCl_2$.

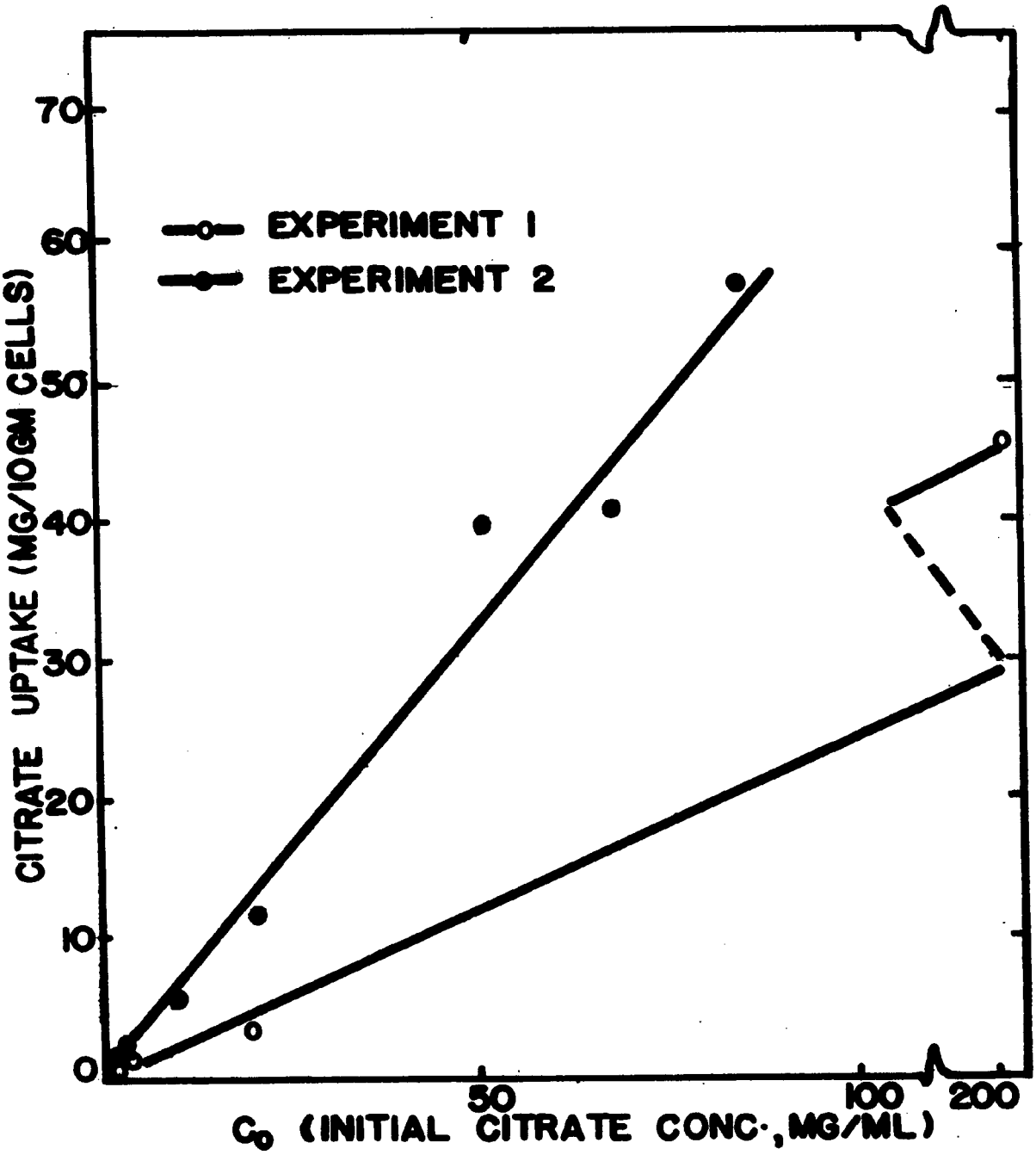


Figure 12. The effect of citrate concentration on uptake of citrate by E. coli. Replot of Figure 11.

responsible for the citrate uptake and that citrate enters the pack in direct response to the external concentration. It is necessary, however, to qualify this statement somewhat in that cells may adsorb very small quantities of citrate as suggested by the fluctuation in space values, as shown in Figure 11.

(g) The effect of ion concentration and species

It has been noted frequently in studying pooling of amino acids in various systems[12] that the rate and amount of amino acid penetration is dependent on the molarity and species of cation present. An experiment was therefore undertaken to determine whether the ionic environment had an effect on citrate space. Cells were grown in the steel fermentor, washed once with gelatine saline solution (0.9% NaCl and 1.0% gelatin), once in distilled water, and once in the desired ionic solution. Table 17 and Figure 13 show the effect of various concentrations of KCl and NaCl on the citrate space of these cells. As the ionic concentration increases there is an increase in citrate space, reaching a maximum at about 0.100 M

NaCl or KCl. Cells without the addition of ions showed a very low space value.

When calcium was used in place of the monovalent cations, the concentration required for the maximum citrate space dropped to 0.050 M as shown in Table 18 and Figure 14. However it was observed in the controls of the same experiment that an increase in NaCl concentration only slightly affected the citrate space. The only apparent difference in these cells from those used for the experiment presented in Table 17 and Figure 13 was that the latter had been washed in gelatin saline solution. The difference in the effect between calcium and sodium suggested that a small amount of calcium was necessary for the maximum effect to be produced and that this was supplied in the initial experiment by the suspected contamination of the gelatin with calcium. When pre-treatments were compared, it was found that the effect of NaCl concentration was greatest in cells which had been previously washed with 0.05 M $CaCl_2$. Washing with gelatin saline gave a lesser effect and water alone

TABLE 17

THE EFFECT OF IONS ON THE CITRATE SPACE OF *E. COLI*: INITIAL OBSERVATION

Cation	Anion	M-Cation	M-Anion	R^1
Na^+, K^+	$Cl^-, HPO_4^=$	0.127	0.110	39.2
Na^+	Cl^-	0.077	0.077	36.1
Na^+, K^+	$Cl^-, HPO_4^=$	0.061	0.055	28.0
K^+	Cl^-	0.061	0.061	26.4
Na^+	Cl^-	0.038	0.038	27.6
K^+	Cl^-	0.030	0.030	15.0
-	-	0.000	0.000	13.0

[1] Temperature 0^o, pH 6.0; citrate concentration 2 mg/ml

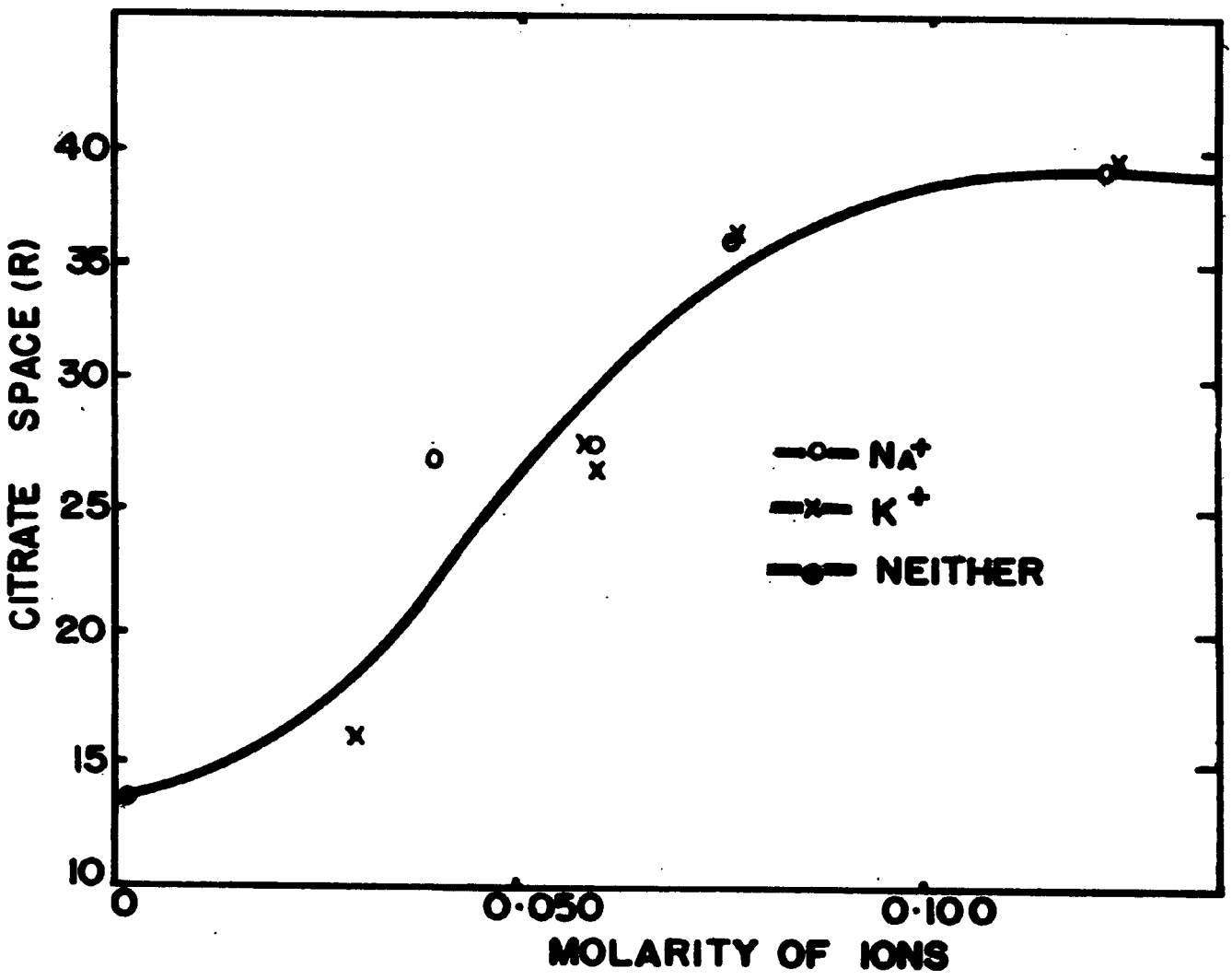


Figure 13. The effect of ion concentration on the citrate space of E. coli: Initial observation.

TABLE 18

THE EFFECT OF IONS ON THE CITRATE SPACE OF *E. COLI*: A COMPARISON OF Ca^{++} AND Na^{+}

Cation	Anion	M-Cation	M-Anion	pH	R
Na^{+}	Cl^{-}	0.100	0.100	4.8	24.9
Na^{+}	Cl^{-}	0.050	0.050	4.9	22.3
Na^{+}	Cl^{-}	0.025	0.025	5.0	21.7
Ca^{++}	Cl^{-}	0.100	0.200	4.8	41.0
Ca^{++}	Cl^{-}	0.050	0.100	4.8	39.8
Ca^{++}	Cl^{-}	0.025	0.050	4.8	30.9

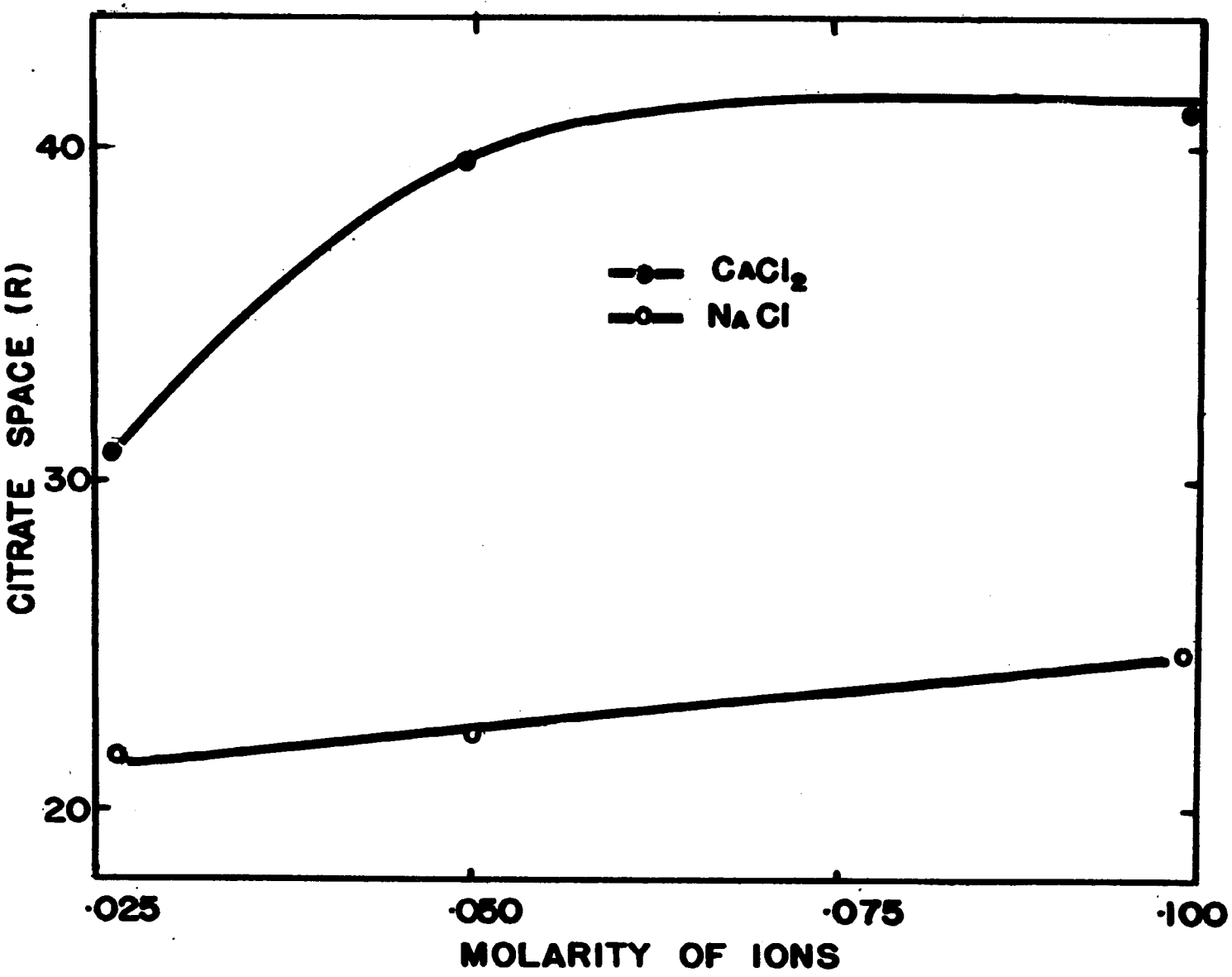


Figure 14. The effect of ion concentration on the citrate space of E. coli.

gave the least response. The data from this experiment are given in Table 19.

The results in Tables 17 and 18 suggested that either the chloride ion or the charge on the cation was involved, since 0.100 M NaCl or KCl was required to give the same increase as 0.050 M $CaCl_2$. To resolve this, the effect of a divalent and a monovalent anion was compared. Since many calcium salts are quite insoluble, magnesium salts were used. A comparison of $MgCl_2$ and $MgSO_4$ on the citrate space is shown in Table 20. It was observed that the concentration of anion had no effect on the space. From this one might conclude that the change in citrate space with addition of salts probably is due to the concentration of positive charges, rather than the concentration of cations since one divalent cation will substitute for two monovalent cations.

This raised the question of the effect of trivalent cations. Accordingly, a series of concentrations of $FeCl_3$ was prepared and tested. The high concentrations of $FeCl_3$ caused a marked drop in the pH of the cells but

TABLE 19

THE EFFECT OF PRE-TREATMENT OF *E. COLI* ON CITRATE SPACE IN THE PRESENCE OF VARYING CONCENTRATIONS OF SODIUM CHLORIDE

Molarity of sodium chloride	Citrate Space (R)		
	Wash with 0.05 M $CaCl_2$	Wash with gelatin saline	Wash with distilled water
0.025	18.0	10.0	20.0
0.050	23.0	12.0	21.5
0.100	29.0	18.0	24.0

TABLE 20

THE EFFECT OF VARYING CONCENTRATIONS OF $MgSO_4$ and $MgCl_2$ ON THE CITRATE SPACE IN *E. COLI*

Salt	Molarity	pH	R
$MgSO_4$	0.100	5.2	26.8
$MgSO_4$	0.050	5.3	20.8
$MgSO_4$	0.025	5.3	18.3
$MgSO_4$	0.012	5.5	23.2
$MgCl_2$	0.100	5.4	29.7
$MgCl_2$	0.050	5.4	24.5
$MgCl_2$	0.025	5.3	20.5
$MgCl_2$	0.012	5.5	15.2

TABLE 21

THE EFFECT OF $FeCl_3$ ON THE CITRATE SPACE OF *E. COLI*

Salt	Molarity	pH	R	% Recovery on washing
$FeCl_3$	0.050	2.5	-	0
$FeCl_3$	0.025	3.7	-	0
$FeCl_3$	0.012	4.6	535	3.1
$FeCl_3$	0.006	5.0	80.5	11.0
$CaCl_2$	0.05	5.2	23.6	65.0
$CaCl_2$	0.025	5.2	24.7	117.0
$CaCl_2$	0.012	5.4	20.4	160.0
NaCl	0.050	5.6	13.0	?

not enough to explain the complete disappearance of the citrate shown by the data in Table 21. At concentrations of 0.050 M and 0.025 M $FeCl_3$ no citrate was recovered in the supernatant nor could it be recovered by washing the cells. At 0.006 M still less citrate was lost and 11% of this was recovered on washing. The loss of citrate may have been due to an adsorptive process, but this does not explain the failure to recover at least some by washing. Another possible explanation is in a stimulation of aconitase action, resulting in the complete loss of citrate. Aconitase is known[59] to require Fe^{++} for its activation. Manometric studies with citrate and added $FeCl_3$ showed no increased rate of oxygen uptake over the endogenous respiration but this does not preclude the activity of aconitase.

(h) The effect of drying on the cells

Since it was shown that dried cells were able to oxidize citrate at an appreciable rate (Figure 7) it was of interest to determine what effect this drying had on the citrate space. If it was the result of disruption

of permeability barriers, as has been suggested by Lara and Stokes[48], then one might expect nearly all the cell water (about 75% of the cell) to be available to the citrate and consequently a citrate space of about 75%.

Cells were prepared in the usual manner after rehydration and the inulin space (intercellular space) and citrate space were determined. The results are shown in Table 22. There is some increase in citrate space as a result of drying although not the degree one would expect for complete permeation. This suggests that the cells on drying do not lose a great deal of their citrate impermeable volume, although they are now fully able to degrade exogenously added citrate via the tricarboxylic acid cycle[1].

(i) <u>The effect of butanol and various other solvent treatments of E. coli on the citrate space</u>

The above experiment made it desirable to determine whether other treatments that increased citrate space would also enable oxidation to take place. It was shown previously that although decreased pH caused an increase

TABLE 22

THE CITRATE SPACE OF DRIED *E. COLI* CELLS

Wp(gm)	Vs(ml)	Co(mg/ml)	Cf(mg/ml)	Sin	R
9.26	8	2.000	1.214	17.7	46.5
8.31	8	2.000	1.212	29.1	47.0

in citrate space, no increase in oxidation resulted. This may, however, have been due to the fact that at low pH, the enzymes were no longer active. Various workers[28,55] have reported that solvents such as butanol or toluene effectively remove certain impermeable barriers in various organisms. Cells were therefore treated with butanol for 5 min and then analyzed for citrate space (0.011 M citrate, pH 6.0, 0°). The results are shown in Table 23. It was found that the space could be increased to about 65% by this method. Previous work (B-3) showed that there is no increased oxygen uptake in the presence of citrate by butanol treated cells, so that increasing the space and presumably breaking the permeability barriers had no effect on the oxidation of citrate.

However, when the citrate space determination was made at 37°, the citrate space increased to a value greater than two, strongly suggesting that the citrate was being acted on metabolically. This may have been

TABLE 23

THE EFFECT OF VARIOUS SOLVENTS AND FREEZING ON CITRATE SPACE OF E. COLI MEASURED AT 0° AND 37°

Treatment	Temperature (°C)	pH	R
			52.0
			78.0
Butanol	0	-	66.0
Butanol	37	-	250.0
Chloroform	0	5.8	43.4
Chloroform	37	6.2	46.7
Toluene	0	5.9	30.6
Toluene	37	6.0	89.4
Freezing[1]	0	5.6	27.5
Freezing[1]	37	5.8	29.8

[1]Quick frozen in dry ice and acetone, thawed in 37° water bath

by virtue of a splitting enzyme such as citritase or a hydrolyzing enzyme such as aconitase.

Chloroform and toluene also were tested at the two temperatures. Chloroform showed a slight space increase, but no response to temperature. Toluene, on the other hand, showed no space increase at 0^{o}, but a considerable one (to 30%) at 37^{o}, suggesting metabolism. Thus it may be possible to separate penetration of citrate from citrate degradation. In this latter example some system may have been activated which can act on citrate at temperatures above 0^{o} and which does not necessarily depend on increased penetration of citrate into the cell. This assumes that penetration occurs equally well at 0^{o}.

In contrast to effects obtained by Foulkes[28] with yeast, freezing the cells caused no change in space available for citrate penetration.

(j) <u>The uptake of citrate by cell particles</u>

It has been suggested by Conway and Downey[15,24] that the cell wall of yeast accounts for most of the apparent uptake of certain substrates by this organism.

The possible adsorptive properties of the cell walls of E. coli made it desirable to determine whether citrate is taken up to any extent by cellular particles, which were thought to be mostly cell wall derivatives. A citrate space of 49% was found for these particles. If one assumes that the cell wall of E. coli accounts for about 20% of the total cell volume, then the space (49%) of the cell wall would approximate 10% of the whole cell volume, a value considerably lower than that usually found for the citrate space of E. coli (20%). The validity of this deduction rests with the actual volume of the cell wall, for if the cell wall comprised for instance 40% of the cell (as seems to be the case in some organisms) then a 20% space is completely accounted for by the cell wall.

3. Attempts to obtain citrate utilizing mutants of E. coli

It would be very useful for purposes of comparison to non-citrate utilizing strains of E. coli, if a mutant that was able to oxidize citrate could be obtained. Such mutants have been reported[46,66,101], although only

certain strains appear to be able to form citrate oxidizing mutants. Mass inoculation of citrate agar plates did not yield any citrate oxidizing colonies. Some minute colonies of E. coli were formed which gave an acid rather than an alkaline reaction on Simmons citrate agar. Although these minute colonies could be transferred from plate to plate, they showed no increased ability to grow on the citrate agar. The identity of these colonies with E. coli was checked by their sensitivity to bacteriophage T_2.

Since this transfer method failed to produce citrate-oxidizing colonies it was thought that perhaps an energy source was necessary. Accordingly succinate, aspartate, acetate, glutamate, glucose and peptone were separately added to Koser citrate broth at 0.1% concentration. These tubes were inoculated with E. coli and incubated for several days at 37°. In all cases, turbidity developed as the cells grew on the non-citrate carbon. Cell samples were periodically streaked on Simmons citrate agar and examined for citrate oxidizing mutants;

none were ever found, although the small acid forming colonies always appeared. At the end of several weeks the cultures were analyzed for citrate content. In all cases, there was about 20% disappearance of the citrate, suggesting that it had been degraded in some way. This is in agreement with the results found by Vaughn et al.[95] and Lomniski, et al[50].

The failure of E. coli, strain B, to form citrate oxidizing mutants suggests that the mechanisms necessary would require more than one mutation per cell, or that the necessary mutation is lethal. It is in agreement with the observations of Koser[44,45,46] and Parr[66] who noted that some strains of E. coli never form citrate oxidizing mutants.

DISCUSSION

Although it was originally supposed that the citrate oxidase system was governed by similar factors in E. coli and B. abortus, it now appears from a comparison of data from both systems that there are basic differences. Therefore, these two organisms will be discussed separately.

I. The block to citrate oxidation in B. abortus

The use of B. abortus to study the block to citrate oxidase in cells was based on the premise that intact B. abortus does not oxidize citrate. However the experimental evidence presented here generally does not support this premise. Although critical experiments that would give a finite answer to the problem have not been carried out, there are a number of lines of evidence that strongly suggest that citrate is slowly oxidized by B. abortus.

It will be observed from a careful scrutiny of Tables 1 to 7, that there is a slight but consistant increase in $QO_2(N)$ in the presence of citrate over that

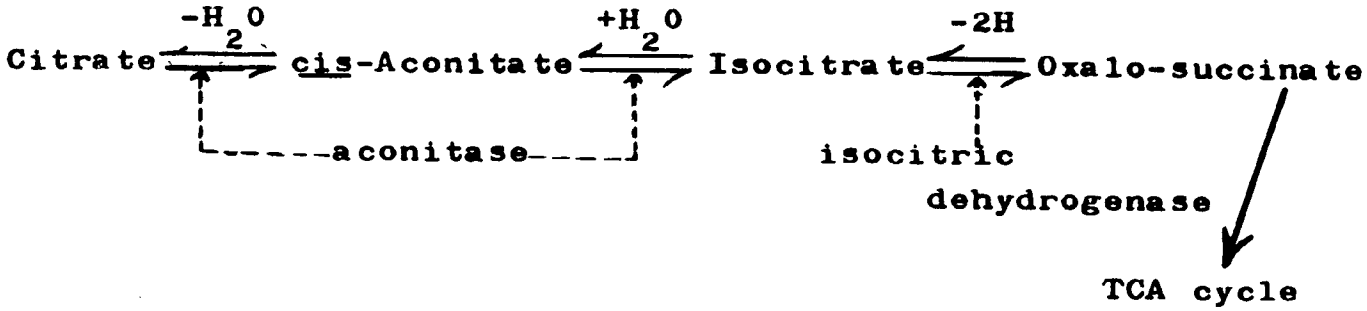
of the endogenous respiration. Although this difference is too small to be considered significant proof of oxidation, by itself, other evidence supports it. For instance when the cell concentration was increased about ten-fold and the endogenous respiration was suppressed by aeration for several hours, a much more significant increase over the endogenous rate was found in the presence of citrate (Table 5). The failure to reproduce this rate increase without reducing the endogenous respiration may possibly be explained on the basis of a non-additive endogenous respiration in the presence of citrate. Some systems have been reported[2,71,79,92,96] in which endogenous respiration is suppressed by substrate, and others in which the endogenous is unaffected by the addition of substrate[9,14,36,62,67,76]. In general, the latter seems to be the more commonly found.

Studies on extracts of these same cells (Figure 4) suggest that the enzymes responsible for citrate oxidation are present only in very limited quantities since $QO_2(N)$ values of from only 40 to 50 were obtained with sonically

disrupted cells. It is possible that the methods employed destroyed much of the enzyme present, but similar preparations from E. coli have given much higher rates of oxidation. This is in agreement with the studies on high cell concentrations and may explain why variously treated cells (e.g. drying, freezing, lyophilized solvent-treatment, etc.) did not cause any increase in oxidation with citrate. That is, not enough oxidation took place to be measurable with the concentration of cells being used.

The studies with the three tricarboxylic acids, especially at high cell concentrations indicate that cells oxidize isocitrate with a greater $QO_2(N)$ than cis-aconitate, which in turn is oxidized at a rate greater than citrate. Since isocitrate is readily oxidized (Figures 1 and 2) even at low cell concentrations, isocitric dehydrogenase must be present in adequate amounts. On the other hand if aconitase were present only in very small amounts, the rate of oxidation of citrate would be dependent on the rate of conversion to isocitrate, pro-

bably a two step process. Since aconitate requires only a one step hydration to isocitrate, the oxidation of aconitate would proceed at a higher rate as is shown by the diagram below.



On the basis of the mechanism recently proposed by Speyer and Dickman[85], the observed increase in oxidation in the presence of aconitate is not so easily explainable. They have reported the same enzyme is necessary to convert both aconitate and citrate to an "activated complex" and thence to isocitrate. A second possible enzyme, similar to aconitic hydrase[61], could be postulated which would convert aconitate directly to isocitrate. Such an explanation is not favored however, in view of the fact that the rate of oxidation of aconitate seems to be governed by the same factors as the rate of oxidation

of citrate.

Therefore, it appears that intact cells of B. abortus do oxidize citrate, as had been suggested previously by Altenburn and Housewright[4]. This oxidation is rate limited by the availability or amount of aconitase and is not dependent on permeability or on an inhibitory factor. When precursors such as succinate are added, pyruvate accumulates as an end product[25,26] due to the block at aconitase and is only slowly removed by way of the normal tricarboxylic acid cycle at a rate limited by the amount of aconitase present. Thus both endogenously and exogenously supplied citrate is necessarily limited in its rate of oxidation. As a result increased oxygen uptake is not observed when citrate is tested by ordinary manometric methods using low concentrations of cells, due to the high rate of endogenous respiration normally present in these cells.

II. The block to citrate oxidation in E. coli

In contrast to what was found in B. abortus, an increase in concentration of E. coli did not produce a

measurable increase in oxidative response to added citrate. Moreover, other workers[1] have demonstrated that intact cells neither exchange endogenous citrate for exogenous, nor oxidize exogenous citrate, but that endogenously formed citrate is cycled around the normal Krebs cycle. Thus the premise that intact cells do not oxidize exogenous citrate is well founded.

On the other hand, it has been frequently shown[1,38,48,70] and can be easily demonstrated that disrupted cells oxidize citrate and that this citrate is cycled through the normal Krebs cycle[1,69,89,90]. What then, does cell disruption do that now permits the oxidation of exogenous citrate?

The simplest explanation is that citrate impermeable membranes are disrupted. The data in Tables 12 to 20, however, suggest that a limited amount of citrate enters the cell under normal conditions, although the size of the space entered may not be greater than that taken up by the cell wall. If citrate actually enters the cell, and this seems likely under certain conditions, it would be desirable to revise the hypothesis about impermeable

membranes since the two membranes most commonly designated, the cell wall and cytoplasmic membrane, are at the cell surface. One could visualize some other structures within the cell which in some way act to keep the citrate from the enzymes which degrade it. This barrier may be in the nature of an interface, as at a sol-gel surface, or at a vacuolar surface, or it may be a true membrane equivalent to mitochondrial or nuclear membranes of higher forms. If this were the case, then one would expect some induction of citrate oxidation when the space was increased. However as shown in Figure 10, for instance, the large increases in citrate space caused by pH changes do not result in increased oxidation. This failure could be rationalized as being due to inactivation of the enzymes at the pH used. On the other hand toluene treatment of the cells caused little or no increase in the space at 0^{o} but a large one at 37^{o}, due in all likelyhood to "activation" of the enzymes. The same is true for heating.

Another possibility which cannot be overlooked, is that the cytoplasmic membrane contracts by plasmolysis, as in higher plant cells, on increase of salt concentration. This would give an increased space without any penetration of the cytoplasmic membrane. Although such a phenomenon has never been observed cytologically in intact bacteria, protoplasts do act in this manner[56]. This would offer an explanation for the data on ion and citrate concentration, effects which could be the result of an altered osmotic equilibrium. The use of protoplasts would greatly help to clarify both the involvement of the cell wall and the reaction of the cytoplasmic membrane to variables governing the amount of space in a cell. To date this experiment has had to await development of a technique for the preparation of stable protoplasts of gram-negative bacteria.

Perhaps the most conclusive information bearing on the impermeability hypothesis has come from studies with treated cells. Dried cells which are able to oxidize citrate show some increase in space but less so than one

would expect for complete loss of impermeable barriers. On the other hand, butanol treatment does not enable the cells to oxidize citrate (at least as measured manometrically) but a larger space is penetrated (Table 23) than with dried cells (Table 22). When the temperature was increased after butanol treatment there was a marked disappearance of citrate, suggesting some metabolism. With toluene, moreover, as was noted above there was no increase in space at 0^{o} but a considerable one at 37^{o}. This increase was probably due to metabolism, but it is not known whether the enzymes released oxidize citrate or degrade it in an anaerobic manner. If the failure to oxidize citrate is due to non-penetration into the cell, it is difficult to explain why oxidation does not take place when cells are treated with solvents which apparently remove permeability barriers (i.e. give a large space) or why treatments such as drying which only partially remove these supposed barriers (i.e. give about 50% space) enable the cells to oxidize citrate readily.

Foulkes[27] has shown a somewhat similar occurrence in yeast, where he was able to destroy permeability barriers (as measured by the conventional space methods) by freezing, but to obtain no increased oxygen uptake in the presence of citrate. If he treated these cells which had been frozen with chloroform they were able to oxidize citrate although there was no change in the "citrate space" of the cells.

Thus, there seems to be little correlation between citrate space and citrate oxidation, suggesting that our present concept of permeability barriers as excluding structures is doubtful.

It should, however, be pointed out that the arguments used here to refute permeability as an explanation are not necessarily valid if a much greater degree of adsorption takes place than is estimated. It is possible, furthermore, that in many of the studies the cells were no longer viable. Unfortunately at the concentrations of cells being used a 75% loss of viability could not be detected due to statistical error of plate counting.

While this may not necessarily affect the citrate oxidase system, too little is known about loss of viability to be sure.

In place of a complicated explanation involving the conventional concepts of permeability, it is somewhat more attractive to attribute this phenomenon to alteration of the enzymes themselves. This concept involves two possibilities: (1) the removal of an inhibitor, and (2) the activation of enzyme molecules that were inert due to a structural arrangement.

The first possibility has been demonstrated in the case of DPN pyrophosphatase in Proteus vulgaris by Swartz, Kaplan and Frech[88]. They showed the presence of a heat labile protein which was able to inactivate the enzyme, but which was inactivated by heat much more rapidly than was the enzyme. Since both the inhibitor and enzyme were readily extractable from the cells by sonic disruption, permeability was not implicated. The present findings on heat "activation" (Figure 7) can be interpreted in the light of this study by Swartz et al.

A possible complicating factor, however, is the extremely narrow shoulder on the enzyme inactivation curve as compared to the endogenous inactivation curve. It appears that, if an inhibitor of citric oxidase is present, this inhibitor is not much less stable than the citric oxidase itself. This could explain why some of the dried cell preparations were found to be less active than others, especially if all the inhibitor was not destroyed by drying. It should, moreover, be possible to remove the inhibitor by other means, if its presence can be established. As yet the evidence for its presence is circumstantial.

The second possibility of enzyme alteration or structural activation is based on the work of Kaplan et al.[29,40,41,42,43], Bonner[5] and Rotman[74], all of whom have found that various treatments of certain cell enzymes will increase their activity many-fold. Rotman has suggested that an enzyme (beta-galactosidase of E. coli) RNA complex is responsible for the decreased activity of untreated cells, but has not been able to

actually demonstrate such a complex. An extensive investigation of the phenomenon of enzyme alteration has been made by Kaplan *et al.*[29,40,41,42,43] with yeast catalase. They have been able to show that treatment of yeast with various solvents (such as butanol, chloroform, or propanol) greatly increases the amount of active cell catalase. There is a loss of heat stability concurrent with this activation, suggesting the disruption of stabilizing chemical bonds. The possibility of an inhibitor was partially ruled out in that the activated catalase could be adsorbed on an oil-water emulsion with a subsequent return to its former "natural" stability and decreased activity. This can also be effected with certain RNA solutions. They have suggested that the catalase in the yeast cell is normally adsorbed at some interface; upon solvent treatment it is desorbed, thereby becoming much more active but with loss of stability.

However it is not immediately apparent that there is any basic difference between the enzymes adsorbing on a lipid interface (Kaplan), on a RNA molecule (Rotman,

Kaplan), or on a protein molecule (Swartz et al). In all cases, certain highly active groups of the enzyme are tied up. Their liberation increases the activity of the enzyme but may also cause the molecule to become more unstable.

An enzyme alteration of this general type is quite possible in the case of citric oxidase in E. coli. The possibility is supported by the disappearance of citrate at increased temperatures after solvent treatment (Table 23); the only problem in this interpertation is that oxidation, even at high concentrations of citrate, cannot be shown by this method. It may be that aconitase is "activated" by treatment, but that isocitric dehydrogenase either is not or is inactivated by it. This would cause the loss of citrate but produce no oxygen uptake. The other possibility is that a citrate splitting enzyme[19,83,99] is activated and the citrate is split anaerobically; this may not occur rapidly enough for the oxidation of the end-products to be distinguished from the endogenous respiration.

The heat activation studies (Figure 7) offer better evidence for the activation of citric oxidase. The heat may cause certain low energy bonds to break in the enzyme molecule, thus desorbing it from an interface and allowing it to express full activity. In this state, however, it is very heat sensitive and so is rapidly inactivated as is shown in Figure 7.

The requirement of the enzyme aconitase for a cysteine-Fe^{++} complex suggests that perhaps by virtue of these cysteine-binding bonds the enzyme is held in an inactive state to some large cysteine protein or polypeptide. It may be of interest to test this hypothesis by adding back some of the cell protein to an active aconitase preparation.

SUMMARY

The apparent inability of intact cells of Brucella abortus and Escherichia coli to oxidize citrate, whereas cell extracts do oxidize it, has been frequently noted and is representative of a general phenomenon of apparent enzyme latency in situ. The present study attempts to explain this anomaly.

The oxidative response of B. abortus to citrate under varying conditions was investigated by means of conventional manometric techniques. It was found that neither varying pH and concentration of citrate, addition or removal of metallic ions, use of citrate analogs, freezing, drying, nor treatment of cells with various solvents had any effect in enabling the cells to measurably increase their rate of oxygen uptake in the presence of citrate. However, cell extracts did contain an active citric oxidase and large masses of cells gave a measurable increase in the rate of oxidation of citrate. Furthermore, isocitric acid lactone appeared to be oxid-

ized readily. It was concluded that citrate is degraded by intact B. abortus, although at a rate too low to be detected by ordinary methods in the presence of its normally high endogenous rate of respiration.

The oxidative response of E. coli to citrate was not altered by the addition of variously substituted esters of citric acid, nor by treatment of the cells with various solvents. However, cell rupture, drying, treatment with lysozyme, or heating for short periods at 100°C revealed an active citric oxidase in E. coli.

The penetration of citrate into B. abortus and E. coli was measured by a direct method which is based on the theory that large masses of cells will act as a diluent for any substrate which penetrates them and that the degree of penetration will be directly proportional to the dilution. Thus,

$$Scit = \frac{Vs}{Wc} \left(\frac{Co}{Cf} - 1\right) \times 100$$

where Scit = percentage of cell pack penetrated by citrate, Vs = volume of substrate, Wo = weight of packed cells, Co = initial concentration of substrate, and Cf =

final concentration. The volume of the intercellular space was determined by a similar formulation using a non-penetrating substrate (inulin) and was substracted from Scit to give the corrected citrate space (R). Citrate was analyzed by a pentabrom acetone method and inulin, gravimetrically.

Thus, it was calculated that citrate entered 41% of a B. abortus cell (in 10 min at pH 6.4 and 0°C) and 23% of an E. coli cell under the same conditions. The space in E. coli penetrated by citrate was not changed by the addition of glucose or isocitrate nor did it decrease with increased citrate concentration. The space is subject to variables of temperature (0° = 23%; 60° = 40%), pH (pH 9.0 = 15%; pH 3.0 = 70%), and ion concentration. In the absence of ions, the space penetrated was 12%, but this increased with ion concentration up to a maximum (40%) in 0.100 M sodium or potassium ions. Calcium or magnesium had a maximal effective concentration of 0.050 M. If present at a concentration over 0.0125 M, ferric ions caused complete disappearance

of the added citrate. A small amount of calcium appeared to be essential for maximum penetration of citrate. Dried cells had 50% of their volume available to citrate. Treatment of cells with butanol, chloroform, or toluene similarly caused a marked increase in citrate space (up to 75%).

It was concluded that the inability of intact E. coli to oxidize exogenous citrate occurs either because of impermeable membranes interior to the cell surface, or because of a labile inhibitor of the oxidase within the cell.

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