PERMEABILITY OF AZOTOBACTER TO SUCCINATE AND MALATE^{1, 2}

ROY REPASKE,³ JOAN SHROAT, AND DAVID ALLMAN⁴

Department of Bacteriology, Indiana University, Bloomington, Indiana

Received for publication August 3, 1959

Determination of metabolic pathways in whole cells by the technique of simultaneous adaptation can be misleading unless, as Stanier (1947) cautioned, one is aware of permeability problems. It is now apparent that cell permeability to postulated intermediates cannot be tested by demonstrating that cells metabolize the substrates after having been specifically adapted to them, since this adaptation may reflect synthesis of specific permeases rather than enzymes directly involved in the metabolism of the components. For example, a β -galactoside permease is required for the permeation of β -galactosides in *Escherichia coli* (Cohen and Monod, 1957), and others have presented evidence that permeases are required for penetration of certain tricarboxylic acid cycle intermediates in pseudomonads (Barrett *et al.*, 1953; Kogut and Podoski, 1953; Clarke and Meadow, 1959). Prior to the demonstration of specific permeases, Karlsson and Barker (1948) concluded that *Azotobacter agilis* did not metabolize acetate by the tricarboxylic acid cycle since the cells were not simultaneously adapted to other intermediates of the cycle; in addition, equilibration of intracellular intermediates with added extracellular pools did not occur. Subsequently, Repaske and Wilson (1953) demonstrated that extracts of this organism contained all of the necessary tricarboxylic acid cycle enzymes, and these were dependent upon the usual cofactors for activity.

¹ This investigation was supported in part by a research grant (E-1905) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

² Preliminary results of this work were presented at the 1958 Society of American Bacteriologists meeting at Chicago, Illinois.

³ Present address: Section on Medical and Physiological Bacteriology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

⁴ Present address: Department of Biochemistry, Indiana University Medical Center, Indianapolis, Indiana.

These results and the data of Stone and Wilson (1952) on *Azotobacter vinelandii*, of Campbell and Stokes (1951) on *Pseudomonas aeruginosa*, and of Swim and Krampitz (1954) on *E. coli* infer that the inability of unadapted cells to oxidize certain tricarboxylic acid cycle substrates immediately is a consequence of permeability deficiencies. The observation that adaptation in cryptic cells is inhibited by ultraviolet light and by amino acid analogues suggested to Barrett *et al.* (1953) and Kogut and Podoski (1953) that adaptation involves the synthesis of a membrane transport or carrier system.

The results reported in this paper show that adaptation to succinate does not involve a significant increase in intracellular succinic oxidase or succinic dehydrogenase of *A. agilis*. Nevertheless, chemical and physical agents which interfere with deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein synthesis prevent adaptation in this system as they do in the more thoroughly studied adaptive β -galactosidase system (Cohn, 1957). The data provide indirect evidence that adaptation to succinate and malate reflects the adaptive synthesis of a permease system.

MATERIALS AND METHODS

Actively growing cultures of *A. agilis* (*A. vinelandii* strain Original) were maintained by daily transfer in Burk's nitrogen-free salts medium containing 3 per cent sucrose. Cultures were grown in 500-ml Erlenmeyer flasks containing 100 ml of medium, and cells were harvested after 16 to 24 hr incubation at 30 C on a rotary shaker. Larger quantities of cells needed for succinic dehydrogenase and succinic oxidase assays were obtained from 8-L mass cultures as previously described (Repaske, 1954).

Cells for manometric studies were washed twice in a total of 50 ml of distilled water except as noted in the text. Following the final wash, cells were resuspended in distilled water to an optical density (600 m μ) of 0.750 (5×10^8 cells

per ml), and 0.5 ml of this suspension was added to the side arm of each Warburg flask. The main compartment contained 100 μ moles tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.0; 30 μ moles potassium phosphate buffer, pH 7.0; 1 μ mole $MgCl_2$, 10 μ moles substrate, pH 7.0; 0.2 mg glucose, and other reagents as required giving a total volume of 3 ml. The center well contained 0.2 ml 20 per cent KOH. Respiration was measured at 30 C in air.

Adapted cells could be obtained either by growing cells with the specific compound as the sole carbon source or by adapting sucrose grown cells. The latter method was used because it provided minimum opportunity for selective enrichment of mutants. During adaptation, no net increase in cell numbers (direct count) was detected. For adaptation, sucrose grown cells harvested by centrifugation were resuspended in sterile distilled water and were added to adapting medium (100 ml of the usual medium used in the Warburg flasks) to a final concentration of 2×10^8 cells per ml. Substrate and glucose were sterilized by Seitz filtration and were added to the sterile buffer and magnesium salt mixture immediately before use. Cells were incubated at 30 C on a rotary shaker for 1 to 2 hr if acetate was the substrate and for 3 hr if other inducers were used. After adaptation, cells were washed twice in distilled water before being diluted to the appropriate concentration for use.

Irradiated cells were exposed to ultraviolet light at a distance of 25 cm from a Hanovia Letheray germicidal lamp. Five ml of a washed cell suspension (5×10^8 cells per ml) were placed in a petri dish cover and swirled by hand during the period of exposure.

Succinic dehydrogenase and succinic oxidase activities (Repaske, 1954) were determined on cell-free extracts prepared from frozen cells obtained by mass culture. Two- to eight-gram quantities of frozen cells suspended in 19 ml of 0.05 M K_2SO_4 were treated for 15 min in a 10 kc Raytheon sonic oscillator. Cell debris was removed by centrifugation at $23,000 \times G$ for 15 min, and extracts were dialyzed against 2 L of 0.05 M K_2SO_4 for 2 hr before testing. Activity was determined with quantities of enzyme which gave a response within the proportional limits of a standard curve. Protein was determined by the biuret method of Gornall *et al.* (1949). Specific activities of succinic oxidase were 2- to

3-fold lower when frozen cells were used instead of freshly harvested cells; succinic dehydrogenase activity, on the other hand, was unchanged or slightly higher in extracts prepared from frozen cells.

Proflavin was obtained from Abbott Laboratories; highest purity stereoisomers of tartaric acid were purchased from Mann Research Laboratories, Inc.; and α,β -dibromosuccinate was purchased from Matheson, Coleman and Bell Division of the Matheson Company.

RESULTS

Figure 1 shows the typical lag period which accompanies adaptation of unwashed sucrose grown *A. agilis* to succinate and malate. It has been repeatedly observed (Karlsson and Barker, 1948; Stone and Wilson, 1952) that *Azotobacter* is adapted to acetate regardless of the carbon

Figure 1. Oxidation of acetate, succinate, and malate by unwashed sucrose grown *Azotobacter agilis*. Each flask contained these reagents at the final concentration indicated: 0.033 M Tris sulfate buffer, pH 7.0; 0.01 M potassium phosphate buffer pH 7.0; 0.0003 M $MgCl_2$, and 10 μ moles substrate in a total volume of 3 ml. The bath temperature was 30 C. Air was the gas phase. Curves are corrected for endogenous respiration.

source used for growth. Adaptation to α -keto-glutarate occurs only in direct response to that substrate, whereas simultaneous adaptation to succinate and malate results when cells are adapted to either of these substrates (figure 3).

Goucher and Kocholaty (1954) and Johnson and Colmer (1957) reported that as a result of washing unadapted *Azotobacter*, the lag period was extended before the cells adapted to succinate. This effect was minimized by using a washing medium containing KCl or phosphate buffer, and the lag returned to normal if Mg^{++} or Ca^{++} was added to the adapting medium. The effect of washing cells twice in distilled water is shown in figure 2; the lag period before oxidation of succinate or malate alone was extended beyond 2 hr, and a 20-min lag period was observed before acetate oxidation (compare with figure 1). The lag period induced by washing was completely eliminated by adding back the cell washings to washed cells. For this purpose it was necessary to combine and concentrate

Figure 2. Oxidation of acetate, succinate, and malate by washed sucrose grown *Azotobacter agilis* and the effect of glucose. Flask contents the same as given in figure 1 except 0.2 mg glucose was also included as indicated. Corrections have been made for endogenous respiration and for oxidation of glucose.

Figure 3. Succinate and malate oxidation by succinate adapted cells. A, 10 μ moles of succinate or acetate used. B, 10 μ moles DL-malate. Other constituents as given in figure 1. Curves have been corrected for endogenous respiration and for glucose oxidation.

cell washings 10-fold by vacuum distillation. As little as 0.05 ml of the concentrated washings per Warburg flask was sufficient to reestablish the original lag period of $\frac{1}{2}$ hr. An excess of the concentrate did not further shorten the lag. A large correction had to be applied because the concentrate contained quantities of readily oxidizable substances; however, it was found that ether extraction removed essentially all of the oxidizable substances without removing the stimulatory factor.

The active substance in the concentrated washings after ether extraction was alkali and acid stable; boiling 10 min in 1 N KOH or 1 N H_2SO_4 or autoclaving 30 min in 1 N H_2SO_4 did not reduce its activity. The 280 $m\mu$ to 260 $m\mu$ absorbance ratio was approximately 0.4 although nucleotides were not responsible for the stimulation since neither Norit A nor Dowex 1 (Cl^-) adsorbed the active component. Amino acids (ninhydrin positive material) present in the crude concentrate were removed by Dowex 50 (H^+) without diminishing the stimulatory effect. The nonionic properties of the active component

(not adsorbed by ion exchange resins), its polar nature (not extracted into ether or other organic solvents), and its stability to acid and alkali suggested the component might be a carbohydrate. Orcinol tests for pentose were negative. Washings from 18-hr cells were anthrone positive (Morris, 1948) and contained 10.4 mg anthrone positive material (calculated as glucose) compared to an estimated 0.7 mg free glucose based on ferricyanide reduction (Park and Johnson, 1949). The reducing sugar value (calculated as glucose) increased to within 5 to 10 per cent of the anthrone results after acid hydrolysis of the washings.

The hydrolyzed material, treated with Dowex 1 (Cl^-), Dowex 50 (H^+), and Norit A, was chromatographed on paper using a 4:1:5 ratio of *n*-butanol-acetic acid-water as solvent (Partridge, 1948); a 1:1 mixture of *p*-anisidine and *p*-aminohippuric acid was used to develop the spots (Block *et al.*, 1955) which were identified by their R_f values and by their colored complexes when viewed under ultraviolet light. Only one spot was detected, and it had the same color and R_f as a co-chromatographed glucose standard.

The identity of glucose as the active component was confirmed in manometric experiments. The lag period preceding succinate oxidation by washed cells was the same whether glucose or concentrated washings were used. Figure 2 illustrates the marked stimulation by glucose on succinate, malate, and acetate oxidation by washed cells. Fructose could be substituted for glucose, but mannose, glucosamine, *N*-acetyl glucosamine, or sucrose had no effect. Maximum reversal of the lag period was obtained with 0.1 to 0.8 mg glucose per 3 ml. At the higher glucose concentrations, glucose oxidation resulted in appreciable oxygen uptake; therefore 0.2 mg glucose per 3 ml was routinely used. It should be noted that occasionally washed cells began oxidizing succinate or malate after 60 to 90 min even though glucose had not been added. This response was correlated with the appearance of gum (increased viscosity) in the cultures; apparently the washing procedures did not remove sufficient endogenous reserves from these cells.

Washed acetate adapted cells like the washed sucrose grown cells do not adapt to succinate or malate unless glucose is added. It was found

that the hexose requirement (glucose or fructose) was not specific because acetate (1 μmole) could be substituted for these sugars when acetate adapted cells were used. Glucose, fructose, and acetate are probably effective because they are readily metabolized and provide a source of energy. After cells have been adapted to succinate or malate, they immediately oxidize these substrates without added glucose and washing has no effect (figure 3). The washing effect, therefore, can be demonstrated only when using substrates to which the cell has not been adapted.

Although whole cells require an adaptive period before metabolizing succinate, succinic oxidase can be demonstrated in cell-free extracts of sucrose grown *A. agilis* (Repaske and Wilson, 1953; Repaske, 1954). This fact may indicate that permeability to succinate is initially limiting in whole cells; however, crude cell-free extracts of sucrose grown cells have less than 10 per cent of the succinic oxidase activity (per mg protein) of whole succinate adapted cells. Therefore, it is necessary to distinguish whether unadapted cells have a limited enzyme content or whether they are impermeable to succinate. If succinic oxidase is limiting in unadapted cells and adaptation represents the synthesis of some necessary component(s) of this system, then the difference in enzyme content should become apparent when the specific activities of succinic dehydrogenase and succinic oxidase are compared in extracts from the two types of cells. Quantities of succinate adapted cells were obtained by growing the cells in 10 L of Burk's medium containing sucrose and succinate (30 and 60 g, respectively). Succinate adaptation was confirmed by manometric tests. Results from several experiments, summarized in table 1, show that the extracts from adapted and unadapted cells had essentially the same specific activities for both succinic dehydrogenase and succinic oxidase. The equivalent enzymatic activity of cell-free extracts, in contrast to the marked difference in initial rates of succinate oxidation by unadapted and succinate adapted whole cells, indicates that adaptation to succinate results in increased accessibility of substrate to the internal oxidase system. Unless a differential loss in enzymatic activity occurred while breaking open the two types of cells, the data are significant even though the activities represent a fraction of the oxidative capacity of

TABLE 1

Succinic dehydrogenase and succinic oxidase activity in cell-free extracts of unadapted and succinate adapted Azotobacter

	2,6-Dichlorophenol Indophenol Reduction, OD ₆₀₀ /Min/Mg Protein	O ₂ Uptake, μ L/Hr/Mg Protein
Unadapted cells	0.221*	6.1†
Adapted cells	0.217	—
	0.199	7.3

Assay conditions are those described by Repaske (1954).

* Average of 3 experiments.

† Average of 6 experiments.

whole adapted cells. These results prompted further studies which supported the tentative conclusion that permeability differences exist in the two types of cells. In these experiments oxygen uptake was used as a convenient though indirect assay of substrate permeability.

Inhibitors. (1) Chloramphenicol:—The response of *Azotobacter* adapting to succinate is typical of a variety of adaptive enzyme responses demonstrated with other substrates in other organisms, and it should be possible to prevent adaptation by the addition of chloramphenicol. Chloramphenicol (30 μ g per ml) completely inhibited succinate adaptation for at least 3 hr when chloramphenicol, substrate, and glucose were added to washed unadapted cells at zero time. If chloramphenicol was added to adapting cells 35, 75, or 125 min after succinate and glucose was tipped, inhibition decreased to 97, 73, and 30 per cent, respectively (figure 4); completely adapted cells were not inhibited by chloramphenicol. These data also show that addition of chloramphenicol to partially adapted cells immediately blocked further adaptation without affecting the permease already formed since the rate of succinate oxidation at the time the inhibitor was added remained constant. Hahn and Wisseman (1951) reported a similar effect of chloramphenicol on lactose adaptation in *E. coli*. Chloramphenicol has been shown to inhibit protein synthesis in *E. coli* and *Staphylococcus aureus* whereas DNA and RNA biosynthesis continued (Wisseman *et al.*, 1954; Gale and Folkes, 1953), but recently Bernlohr and Webster (1958) reported that glycine-C¹⁴ incorporation into both DNA and protein of *A.*

Figure 4. Chloramphenicol inhibition of succinate adaptation by washed *Azotobacter*. In a series of flasks, cells were tipped at zero time into the main compartment which contained succinate and glucose. At the times indicated by arrows, chloramphenicol was added from a second side arm. The final chloramphenicol concentration was 30 μ g per ml. Curves are corrected for glucose oxidation.

agilis was inhibited by the chloramphenicol concentration reported here. Glycine incorporation into DNA and protein is indirect and the site of chloramphenicol inhibition is not known; nevertheless, it is not possible to conclude from these data that chloramphenicol here inhibits adaptation to succinate solely by inhibition of protein synthesis.

(2) Amino acid analogues:—Additional evidence that protein synthesis accompanies succinate adaptation was obtained with amino acid analogues. Succinate adaptation was followed manometrically in the presence of *p*-fluorophenylalanine, allyl glycine, imidazole, *D*-leucine, *D,L*- α -methyl glutamic acid, β -2-thienylserine, and methionine sulfoxide at concentrations of 0.05 to 0.2 mg per ml. Of these, only *p*-fluorophenylalanine caused appreciable inhibition at 200 μ g per ml (table 2). The first experiment shown in table 2 also compares the effect of *p*-

TABLE 2
Effect of various agents on succinate
oxidation

	Unadapted Cells		Succinate Adapted Cells	
	O ₂ uptake	Change	O ₂ uptake	Change
	$\mu\text{L}/120$ <i>min</i>	%	$\mu\text{L}/50$ <i>min</i>	%
I. Control.....	439	—	394	—
Proflavin (10 $\mu\text{g}/$ ml).....	9	-98	316	-20
p-Fluorophenyl- alanine (200 $\mu\text{g}/\text{ml}$).....	211	-50	345	-12
8-Azaguanine (200 $\mu\text{g}/\text{ml}$).....	146	-67	363	-8
II. Control.....	366	—		
NH ₄ Cl (50 $\mu\text{g}/$ ml).....	363	0		
Adenine-SO ₄ (100 $\mu\text{g}/\text{ml}$).....	473	+19		
NH ₄ Cl + ade- nine-SO ₄	493	+35		
III. Control.....	459	—		
5,6-Dichloro-1- β -D-ribofurano- sylbenzimidaz- ole				
10 $\mu\text{g}/\text{ml}$	425	-7		
20 $\mu\text{g}/\text{ml}$	302	-34		

* Refer to figure 1 for assay conditions.

fluorophenylalanine, 8-azaguanine, and proflavin on succinate oxidation by unadapted and succinate adapted cells. As with chloramphenicol, adapted cells are resistant to the inhibitors.

Since *Azotobacter* fixes nitrogen, adaptation can occur in the absence of added amino acids, purines or pyrimidine bases; however, it might be possible to bypass nitrogen fixation and promote adaptation by supplying a form of fixed nitrogen. Ammonium chloride, as shown in table 2, had no effect on succinate adaptation. Adenine sulfate (or adenine) stimulated adaptation by 19 per cent, and adenine sulfate and ammonium chloride together stimulated by 35 per cent. The increased oxygen uptake was a result of a shortened lag period; no significant increase in the rate of succinate oxidation was observed. Thymine, uracil, cytosine, guanine, and guanine sulfate had no effect singly or in combination.

Except for glycine which inhibited adaptation, added amino acids had no effect.

(3) Proflavin:—The key role of nucleic acid synthesis in adaptive enzyme formation is well documented, and, consequently, it is important to determine if adaptation to succinate is prevented by agents which inhibit nucleic acid synthesis. One inhibitor, proflavin, was shown by De Mars *et al.* (1953) to cause the development of protein "doughnuts" which are abnormal T2 phage devoid of nucleic acid. Subsequently, De Mars (1955) determined the effect of proflavin on DNA synthesis in T2 infected *E. coli* but found no evidence for inhibition of DNA synthesis. Although proflavin causes no gross effect on total DNA synthesis, Astrachan and Volkin (1957) have reported that proflavin does alter the type of DNA found. Normal T2 phage DNA was compared with DNA from P³²-labeled *E. coli*. The two types of DNA were mixed, treated with heat, and the resulting polynucleotides were separated chromatographically. A comparison of the specific activities of various fractions indicated that "phage DNA and DNA isolated from proflavin-inhibited, phage-infected *E. coli* are not the same."

Adaptation to succinate (or malate) is inhibited 24, 40, and 100 per cent, respectively, by 2.5, 5, and 10 μg proflavin per ml, but proflavin has little effect on succinate oxidation by succinate adapted cells (table 2 and figure 7). In another experiment (figure 5), unadapted cells were tipped at zero time into a series of flasks containing succinate and glucose; at various time intervals, proflavin was then added from the second side arm. Again, as with chloramphenicol, the inhibitor immediately blocked further adaptation without interfering with the adaptation which had already occurred.

(4) Ultraviolet light:—Entner and Stanier (1951) and Brandt *et al.* (1951) simultaneously reported that ultraviolet light inhibited adaptive enzyme formation without affecting constitutive enzymes. Barrett *et al.* (1953) and Kogut and Podoski (1953) both demonstrated that ultraviolet light inhibited adaptation of *Pseudomonas* to certain tricarboxylic acid cycle intermediates, but ultraviolet light did not inhibit substrate oxidation by adapted cells. Kelner (1953) and others have shown that ultraviolet radiation results in a specific inhibition of DNA synthesis. Experiments with unadapted and

Figure 5. Proflavin inhibition of succinate adaptation by washed *Azotobacter*. The experimental procedure is the same as described for figure 4 except proflavin at a final concentration of $10 \mu\text{g}$ per ml was used in place of chloramphenicol. Curves are corrected for glucose oxidation.

with succinate or malate adapted *Azotobacter* show that ultraviolet radiation specifically inhibits adaptation without affecting the oxidative system of the cell. Washed sucrose grown (unadapted) cells exposed to ultraviolet light for 65 sec were prevented from adapting to succinate or malate with or without added glucose (figure 6). Adapted cells were completely refractory to this dose of ultraviolet light; and, in fact, no effect on succinate or malate oxidation was observed until the exposure time was increased to 150 sec.

(5) RNA inhibitors:—The involvement of RNA synthesis in adaptive enzyme formation has been established (Pardee, 1954; Spiegelman *et al.*, 1955; Gale and Folkes, 1955) even though the order of appearance of RNA and new DNA is not resolved. Creaser (1956) reported that 8-azaguanine inhibited the adaptive formation of β -galactosidase and catalase in *S. aureus* and concurred with the findings of Lasnitzki *et al.* (1954) that 8-azaguanine was incorporated into RNA but not DNA. 8-Azaguanine was more effective in the *Staphylococcus* system

Figure 6. Effect of ultraviolet light on washed sucrose-grown *Azotobacter*. Total ultraviolet light exposure was 65 seconds. The manometric assay was carried out as described in figure 1. Non-exposed cells tested simultaneously with controls gave the typical response shown in figure 2.

than in the *Azotobacter* system where $200 \mu\text{g}$ per ml was required to inhibit adaptation 55 to 65 per cent (table 2).

Tamm (1957), who investigated RNA biosynthesis in the chorioallantoic membrane of embryonated chicken eggs, found that 5,6-dichloro - 1 - β - D - ribofuranosylbenzimidazole inhibited adenosine-8- C^{14} incorporation into RNA without inhibiting protein synthesis or oxygen uptake by this membrane. The data in table 2 show that this agent was an effective inhibitor of succinate adaptation in *Azotobacter*.

(6) Inhibitors of phosphorylation:—2,4-Dinitrophenol, sodium azide, and arsenate, agents which uncouple oxidative phosphorylation, were tested with succinate adapted cells to determine if succinate permeation required an internal source of energy. Two controls were used to confirm succinate adaptation by these cells: (a) It was shown that these cells oxidized succinate immediately, and (b) It was shown that proflavin which inhibits adaptation, had no effect on the rate of succinate oxidation (figure 7A and B). Succinate oxidation was not inhibited by 2,4-dinitrophenol or by sodium

Figure 7. The effect of oxidative phosphorylation uncoupling agents on succinate oxidation in succinate adapted cells and protoplasts made from these cells. *Azotobacter* was adapted to succinate in the usual way, and a sample of cells was converted to protoplasts by lysozyme-Versene treatment and washed. Final succinate concentration in all flasks was 3.3×10^{-3} M. Other reagents: proflavin 10 μg per ml; 2,4-dinitrophenol, 1×10^{-4} M; Na azide 1×10^{-3} M; Na arsenate, 0.01 M (phosphate omitted). Curves corrected for endogenous respiration.

azide, but when arsenate replaced phosphate in the reaction mixture, a 50 per cent decrease in the rate of succinate oxidation was observed (figure 7A). A sample of these cells was converted to protoplasts by treatment with lysozyme and Versene (Repaske, 1958), and dinitrophenol and azide inhibition was again determined (figure 7B). The curves show that succinate oxidation in whole cells and in protoplasts is essentially the same with or without added proflavin. Azide (1×10^{-3} M) and 2,4-dinitrophenol (1×10^{-4} M), which had no effect on succinate oxidation by whole cells, inhibited succinate oxidation in protoplasts by 30 and 80 per cent, respectively. Uncoupling agents would not be expected to affect oxidative reactions (Repaske and Josten, 1958) unless substrate activation were required, and the fact that the initial rate of succinate oxidation is

inhibited by dinitrophenol suggests that succinate permeation is dependent upon a source of energy. These data point out an ancillary fact, namely that dinitrophenol and azide do not freely permeate intact cells of *Azotobacter*.

Inducers. Simultaneous adaptation to succinate and L-malate occurred when either substrate was used as inducer. This suggests that only the 4 carbon dicarboxylic acid structure is necessary to initiate the formation of the permease(s) because the additional hydroxyl group on malate does not restrict the cells to adaptation to malate. Other compounds were tested to determine the relationship of structure and stereoisomerism to the ability of a compound to function as an inducer of succinate or malate permease. Tartaric acid was selected because three stereoisomers are available.

Sucrose grown cells were adapted to each of the three forms of tartaric acid by the same proce-

Figure 8. Adaptation to succinate and malate by succinate and D-tartrate adapted cells. Sucrose grown *Azotobacter* was incubated in adaptation media containing either potassium succinate or D-tartrate. After washing, adaptation to succinate and malate was tested manometrically. Ten μmoles substrate were used; other flask contents are given in figure 1. All curves are corrected for endogenous respiration.

Figure 9. Adaptation to succinate and malate by *meso*-tartrate and L-tartrate adapted cells. Cells were adapted to *meso*-tartrate or L-tartrate and assayed for activity as indicated in figure 8. All curves are corrected for endogenous respiration.

ture used for adapting cells to succinate. After 3 hr induction, the cells were washed, and adaptation to succinate and malate was determined by measuring succinate and L-malate oxidation manometrically. Figures 8 and 9 compare the capacity of succinate, D-tartrate, L-tartrate or *meso*-tartrate to induce adaptation to succinate and malate. *meso*-Tartrate adapted cells immediately oxidized succinate and malate at equal rates; D-tartrate, on the other hand, preferentially induced adaptation to succinate, whereas L-tartrate preferentially induced adaptation to L-malate. Although the inducers did not cause exclusive adaptation to succinate or malate, these results provided the first evidence that individual systems for succinate and malate permeation may exist.

Neither *meso*-tartrate nor L-tartrate were metabolized by adapted cells although D-tartrate was metabolized. Apparently D-tartrate adaptation is readily lost because the cells were shown to adapt again in the Warburg flask. The instability of this system is similar to that reported for *Pseudomonas fluorescens* by Shilo and Stanier (1957).

α,β -Dibromosuccinate was also tested for its ability to induce adaptation to succinate. Cells incubated with glucose and dibromosuccinate for 3 hr were adapted to both succinate and malate but were unable to oxidize the inducer. Thus, by using *meso*-tartrate, L-tartrate or α,β -dibromosuccinate, induction of the permease(s) can be shown independently of substrate oxidation.

DISCUSSION

Goucher and Kocholaty (1954) showed that glucose or acetate grown *A. agilis* lost much of their original succinate oxidizing (adapting) capacity as a result of one to five washings in distilled water or in phosphate buffer. The first phosphate washing resulted in a 50 per cent decrease in net oxygen uptake in 2 hr, and subsequent washing caused further loss of activity. Presumably magnesium ions were removed during washing because pre-incubation of cells with magnesium sulfate reestablished the original oxidative capacity of the cell. Johnson and Colmer (1957) also observed a similar magnesium ion effect when cells were washed with KCl, but neither of the above publications reported reactivation of distilled water washed cells by magnesium salts. In the present study, it was shown that under these conditions magnesium is not sufficient to eliminate the extended lag period before succinate adaptation, since, in figure 2, the flask with succinate alone contained $MgCl_2$. A stimulatory substance capable of reactivating washed cells was detected in the cell washings. This substance was hydrolyzed, purified, and identified as glucose by paper chromatography. Glucose had the same biological effect as the purified material. Salt solutions used by various investigators for washing *Azotobacter* may simply minimize leaching of the carbohydrate from the cells.

Sucrose or acetate grown *Azotobacter* are similar in regard to their inability to oxidize succinate or malate without adaptation. Glucose probably is a nonspecific substrate which provides energy for succinate adaptation, for acetate can also replace glucose if acetate adapted cells are used. In an experiment designed to determine if acetate were converted to glucose, large quantities of cells were incubated with acetate and at various intervals of time samples were analyzed for glucose (anthrone test).

No detectable increase in glucose was found. It is unlikely that glucose and acetate provide an intermediate which is necessary for succinate oxidation because washed succinate adapted cells do not require glucose (figure 3), and consequently one would have to postulate a separate mechanism for succinate oxidation by adapted cells.

The inability of unadapted cells to oxidize succinate, whereas succinic dehydrogenase and succinic oxidase activity can be demonstrated in extracts of these cells, suggests that succinate cannot permeate unadapted cells and adaptation corresponds to the adaptive formation of a permease. This is substantiated by the finding that no change in the specific enzyme content occurs during adaptation; succinic dehydrogenase and succinic oxidase activities in extracts of unadapted and succinate adapted *Azotobacter* are the same (table 1).

The data indicate that the permease system cannot be formed if DNA synthesis is inhibited during induction. Proflavin and ultraviolet light, both of which inhibit DNA synthesis, also inhibit adaptation. 8-Azaguanine and 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (table 2) interfere with normal RNA synthesis, and both were shown to inhibit succinate adaptation. The necessity for protein synthesis concomitant with permease formation was indicated by *p*-fluorophenylalanine and by chloramphenicol inhibition. Since none of these inhibitors is effective after succinate adaptation is complete, their actions are not on the functional permease or oxidase but on the formation of the adaptive permease. The effect of these inhibitors on succinate adaptation is typical of the effects observed on other adaptive enzyme systems. Proflavin and chloramphenicol were also shown to be useful for blocking adaptation at any stage of development without affecting the permease already formed (figures 4 and 5).

Succinate permeation in succinate adapted cells would be expected to require energy, but reagents such as 2,4-dinitrophenol or azide, which uncouple oxidative phosphorylation, had little effect on succinate oxidation by whole cells. In contrast, succinate oxidation by protoplasts prepared from these adapted cells was inhibited by both reagents, particularly dinitrophenol. These results indicate that a source of adenosine triphosphate is directly or indirectly

required for succinate permeation because uncoupling agents either have no effect on oxidative reactions or they stimulate oxidation in tightly coupled oxidative phosphorylating systems. No effect of azide or dinitrophenol was found on succinic oxidase (Repaske, 1954) or reduced diphosphopyridine nucleotide oxidase (Repaske and Josten, 1958) in cell-free extracts of *Azotobacter*.

No evidence for individual succinate and L-malate permeases was obtained when either of these substances was used as an inducer since each caused the cells to simultaneously adapt to the other. However, specific permeases are probably required for each substrate in view of the differential adaptation to succinate or L-malate which was found when cells were induced with D-tartrate or L-tartrate (figures 8 and 9). Effective induction of succinate and malate permeases can be achieved with compounds such as L-tartrate, *meso*-tartrate, and α,β -dibromosuccinate which are not metabolized by the cell.

ACKNOWLEDGMENTS

The authors are indebted to Mr. John Berech for making the paper chromatographs of the samples, to Dr. Henry Mahler for the sample of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole, and to Parke, Davis and Company for the gift of chloramphenicol.

SUMMARY

Adaptation of sucrose grown *Azotobacter agilis* to succinate and malate was shown to be inhibited if the cells were washed in distilled water; the normal adaptive lag period of $\frac{1}{2}$ hr was extended beyond 2 hr. A factor found in the washings could be added back to washed cells to reestablish the $\frac{1}{2}$ -hr lag period. This factor was isolated and identified as glucose or a readily metabolized glucose polymer.

Although unadapted cells could not oxidize succinate immediately, cell-free extracts from these cells possessed succinic dehydrogenase and succinic oxidase activities. Extracts from unadapted cells and from succinate adapted cells were compared with regard to these enzyme activities, and the specific activities were the same. Succinate adaptation, therefore, did not involve a qualitative or quantitative change in the succinate oxidizing enzymes; this indicated

that succinate permeation is restricted in unadapted cells.

Adaptation to succinate was prevented if either deoxyribonucleic acid, ribonucleic acid, or protein synthesis was inhibited by such agents as ultraviolet light, proflavin, 8-azaguanine, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole, chloramphenicol, or *p*-fluorophenylalanine. This effect was apparent only on unadapted or partially adapted cells; succinate oxidation was not inhibited after adaptation was complete.

Sodium azide and 2,4-dinitrophenol, agents which uncouple oxidative phosphorylation, were used to demonstrate that permeation of succinate requires an endogenous source of energy. The rate of succinate oxidation by protoplasts prepared from succinate-adapted cells was inhibited 30 and 80 per cent by 1×10^{-3} M azide and 1×10^{-4} M dinitrophenol, respectively. Inhibition by these reagents could not be shown with whole cells, presumably because whole cells were not permeable to azide and dinitrophenol.

REFERENCES

- ASTRACHAN, L. AND VOLKIN, E. 1957 Chromatographic detection of differences between bacteriophage-related deoxyribonucleic acids. *J. Am. Chem. Soc.*, **79**, 130-134.
- BARRETT, J. T., LARSON, A. D., AND KALLIO, R. E. 1953 The nature of the adaptive lag of *Pseudomonas fluorescens* toward citrate. *J. Bacteriol.*, **65**, 187-192.
- BERNLOHR, R. W. AND WEBSTER, G. C. 1958 Effect of chloramphenicol on protein and nucleic acid metabolism in *Azotobacter agilis*. *J. Bacteriol.*, **76**, 233-238.
- BLOCK, R. J., DURRUM, E. L., AND ZWEIG, G. 1955 *A manual of paper chromatography and paper electrophoresis*. Academic Press, Inc., New York, New York.
- BRANDT, C. L., FREEMAN, P. J., AND SWENSON, P. A. 1951 The effect of radiations on galactozymase formation in yeast. *Science*, **113**, 383-384.
- CAMPBELL, J. J. R. AND STOKES, F. N. 1951 Tricarboxylic acid cycle in *Pseudomonas aeruginosa*. *J. Biol. Chem.*, **190**, 853-858.
- CLARKE, P. H. AND MEADOW, P. M. 1959 Evidence for the occurrence of permeases for tricarboxylic acid cycle intermediates in *Pseudomonas aeruginosa*. *J. Gen. Microbiol.*, **20**, 144-155.
- COHEN, G. N. AND MONOD, J. 1957 Bacterial permeases. *Bacteriol. Rev.*, **21**, 169-194.
- COHN, M. 1957 Contributions of studies on the β -galactosidase of *Escherichia coli* to our understanding of enzyme synthesis. *Bacteriol. Rev.*, **21**, 140-168.
- CREASER, E. H. 1956 The assimilation of amino acids by bacteria. 22. The effect of 8-azaguanine upon enzyme formation in *Staphylococcus aureus*. *Biochem. J.*, **64**, 539-545.
- DE MARS, R. I. 1955 The production of phage-related materials when bacteriophage development is interrupted by proflavine. *Virology*, **1**, 83-99.
- DE MARS, R. I., LURIA, S. E., FISHER, H., AND LEVINTHAL, C. 1953 The production of incomplete bacteriophage particles by the action of proflavine and the properties of the incomplete particles. *Ann. inst. Pasteur*, **84**, 113-128.
- ENTNER, N. AND STANIER, R. Y. 1951 Studies on the oxidation of glucose by *Pseudomonas fluorescens*. *J. Bacteriol.*, **62**, 181-186.
- GALE, E. F. AND FOLKES, J. P. 1953 The assimilation of amino-acids by bacteria. 15. Actions of antibiotics on nucleic acid and protein synthesis in *Staphylococcus aureus*. *Biochem. J.*, **53**, 493-498.
- GALE, E. F. AND FOLKES, J. P. 1955 The assimilation of amino acids by bacteria. 21. The effect of nucleic acids on the development of certain enzymic activities in disrupted staphylococcal cells. *Biochem. J.*, **59**, 675-684.
- GORNALL, A. G., BARDAWILL, C. J., AND DAVID, M. M. 1949 Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.*, **177**, 751-766.
- GOUCHER, C. R. AND KOCHOLATY, W. 1954 Effect of various ions on the respiration of *Azotobacter*. *J. Biol. Chem.*, **211**, 613-620.
- HAHN, F. E. AND WISSEMAN, C. L., JR. 1951 Inhibition of adaptive enzyme formation by antimicrobial agents. *Proc. Soc. Exptl. Biol. Med.*, **76**, 533-535.
- JOHNSON, E. J. AND COLMER, A. R. 1957 Further studies on the mode of action of 2,4-dichlorophenoxyacetic acid on *Azotobacter vinelandii* as related to magnesium and phosphate. *J. Bacteriol.*, **73**, 666-669.
- KARLSSON, J. L. AND BARKER, H. A. 1948 Evidence against the occurrence of a tricarboxylic acid cycle in *Azotobacter agilis*. *J. Biol. Chem.*, **175**, 913-921.
- KELNER, A. 1953 Growth, respiration and nucleic acid synthesis in ultraviolet-irradiated and in photoreactivated *Escherichia coli*. *J. Bacteriol.*, **65**, 252-262.
- KOGUT, M. AND PODOSKI, E. P. 1953 Oxidative pathways in a fluorescent *Pseudomonas*. *Biochem. J.*, **55**, 800-811.

- LASNITZKI, I., MATTHEWS, R. E. F., AND SMITH, J. D. 1954 Incorporation of 8-azaguanine into nucleic acids. *Nature*, **173**, 346-348.
- MORRIS, D. L. 1948 Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science*, **107**, 254-255.
- PARDEE, A. B. 1954 Nucleic acid precursors and protein synthesis. *Proc. Natl. Acad. Sci. U. S.*, **40**, 263-270.
- PARK, J. T. AND JOHNSON, M. J. 1949 A sub-microdetermination of glucose. *J. Biol. Chem.*, **181**, 149-151.
- PARTRIDGE, S. M. 1948 Filter-paper partition chromatography of sugars. 1. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. *Biochem. J.*, **42**, 238-248.
- REPASKE, R. 1954 Succinic dehydrogenase of *Azotobacter vinelandii*. *J. Bacteriol.*, **68**, 555-561.
- REPASKE, R. 1958 Lysis of gram-negative organisms and the role of Versene. *Biochim. et Biophys. Acta*, **30**, 225-232.
- REPASKE, R. AND JOSTEN, J. J. 1958 Purification and properties of reduced diphosphopyridine nucleotide oxidase from *Azotobacter*. *J. Biol. Chem.*, **233**, 466-471.
- REPASKE, R. AND WILSON, P. W. 1953 Oxidation of intermediates of the tricarboxylic acid cycle by extracts of *Azotobacter agile*. *Proc. Natl. Acad. Sci. U. S.*, **39**, 225-232.
- SHILO, M. AND STANIER, R. Y. 1957 The utilization of the tartaric acids by *Pseudomonads*. *J. Gen. Microbiol.*, **16**, 482-490.
- SPIEGELMAN, S., HALVORSON, H. O., AND BEN-ISHAÏ, R. 1955 *Symposium on amino acid metabolism*. Edited by W. D. McElroy and H. B. Glass. Johns Hopkins Press, Baltimore, Maryland.
- STANIER, R. Y. 1947 Simultaneous adaptation: A new technique for the study of metabolic pathways. *J. Bacteriol.*, **54**, 339-348.
- STONE, R. W. AND WILSON, P. W. 1952 Respiratory activity of cell-free extracts from *Azotobacter*. *J. Bacteriol.*, **63**, 605-617.
- SWIM, H. E. AND KRAMPTZ, L. O. 1954 Acetic acid oxidation by *Escherichia coli*: quantitative significance of the tricarboxylic acid cycle. *J. Bacteriol.*, **67**, 426-434.
- TAMM, I. 1957 Ribonucleic acid synthesis and influenza virus multiplication. *Science*, **126**, 1235-1236.
- WISSEMAN, C. L., JR., SMADEL, J. E., HAHN, F. E., AND HOPPS, H. E. 1954 Mode of action of chloramphenicol. I. Action of chloramphenicol on assimilation of ammonia and on synthesis of proteins and nucleic acids in *Escherichia coli*. *J. Bacteriol.*, **67**, 662-673.